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BACTERIAL WILT RESISTANCE AND GENETIC HOST-PARASITE INTERACTIONS IN MAIZE[1]

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INTRODUCTION

Bacterial variation has been studied mainly in vitro, few investigations having been made on the effect of host passage on the pathogen. The common belief is that virulence is maintained or enhanced by passage through a host. Outstanding as a critical analysis of this problem is the work of Wellhausen (*19*),[3] who determined the effect of repeated passage of *Phytomonas stewartii* (E. F. S.) Bergey et al.[4] through resistant and susteptible lines of maize and through certain other grasses. Successive passage through susceptible maize decreased virulence, while successive passage through resistent maize increased virulence.

This investigation has been devised to check and extend Wellhausen's observations on the effect of the host upon bacterial virulence and to determine the factors that operate to bring about such changes. The same organism, *Phytomonas stewartii*, has been used.

REVIEW OF LITERATURE

Smith (*14*), the first to study *Phytomonas stewartii*, considered it to be very stable in culture. In 1918 McCulloch (*10*) reported two distinct types of colonies, both remaining stable for at least 2 years. Other workers (*5, 6, 22*) report that atypical strains of *Ph. stewartii* may be isolated from infected soil or plants. Recently McNew (*11*) isolated single colonies from dilution plates of virulent cultures of *Ph. stewartii* and found stock cultures to be composed of many variants as regards virulence. Later, working with 10 strains of *Ph. stewartii* differing in virulence, McNew (*13*) found the strains also differed in other physio ogical reactions. Ability to use inorganic nitrogen was correlated positively with virulence. From studies of 22 cultures Ivanoff et al. (*8*) report that *Ph. stewartii* includes several types of organisms separable into 3 primary types on the basis of morphological, physiological, and colony characteristics. Positive correlation between amount of gum and pathogenicity was indicated. A similar correlation between consistency of growth on agar and virulence also was observed by Wellhausen (*19*). The cultural and physiological similarities between cultures of *Ph. stewartii* and *Ph. vascularum* have been noted by Ivanoff (*7*).

[1] Received for publication June 6, 1939. Journal paper No. G–955 of the Iowa Agricultural Experiment Station, Genetics Section, project 404. Taken from a thesis submitted to the faculty of the Graduate College, Iowa State College, in partial fulfillment of the requirements for the degree of doctor of philosophy. The complete thesis is on file in the Iowa State College Library.

[2] The author wishes to express his appreciation to Dr. E. W. Lindstrom and Dr. J. W. Gowen for suggestions and criticism throughout the progress of this work.

[3] Italic numbers in parentheses refer to Literature Cited, p. 238.

[4] The nomenclature of this species is debatable and is here used to designate the nonmotile bacterium of Stewart that is the causal organism of bacterial wilt of maize. It is also known as *Bacterium stewartii* E. F. S. and as *Aplanobacter stewartii* (E. F. S.) McCulloch.

Allen and Baldwin (*1*) determined the effect of repeated plant passage of effective and ineffective strains of *Rhizobium*, finding that effective strains may become ineffective, or ineffective strains may become effective during repeated plant passage

Hendrickson et al. (*4*) passed the crown gall bacterium through the tomato, finding variation in the physiological behavior of the crown gall organism during plant passage, but noting no effect upon virulence.

Webster and Clow (*17*) found that strains of pneumococcus vary widely in virulence. Serial passage from nose to nose consistently failed to raise the internasal virulence of initially low virulent strains; serial internasal passage of strains with high initial virulence reduced internasal virulence almost to zero without inducing any change in the interperitoneal virulence. Earlier, Webster (*16*) had observed that virulent strains of *Bacterium lepisepticum* may be replaced in the nasal passages of rabbit by relatively avirulent strains.

With mouse *Pasteurella*, Greenwood et al. (*2*) found avirulent strains arising by passage through immunized animals, whereas strains obtained from nonimmunized mice were as virulent as the original strain. In one instance, a virulent epidemic of mouse typhoid arose by emergence of a virulent variant from a relatively avirulent strain of *Bacillus aertrycke*.

The criticism of most investigations on host passage is that host resistance has not been kept a constant, hence the cause of any bacterial variation is difficult to interpret.

EXPERIMENTAL MATERIALS AND METHODS

Host material for these virulence studies was limited largely to four lines of maize (*Zea mays* L.) inbred for at least 10 generations. A yellow dent line, OSF, was the resistant host, while a white flint line, WF, and two yellow sweet lines, GB134 and GB797, were used as highly susceptible hosts. GB134 was used in passage work, while GB797 was used for all virulence tests.

Resistant line OSF when inoculated with virulent *Phytomonas stewartii* showed characteristic lesions only on those leaves that were actually expanded at the time of inoculation, subsequent leaves being free from lesions. Stunting of the plant was slight. Susceptible lines GB134 and WF died soon after inoculation, usually within 2 weeks. With these lines the initial symptoms were the characteristic wilt lesions. The bacteria soon became systemic throughout the vascular system, a condition which resulted in a wilting and was soon followed by drying and death. GB797 reacts like GB134 or WF except that it is slightly more resistant and dies somewhat more slowly. Wellhausen (*20*) determined the genetic basis of resistance between these lines as being primarily influenced by three supplementary dominant factors.

Several cultures [5] of *Phytomonas stewartii* were tested for virulence, and from this group 6 cultures that represented a range of virulence from high to low were selected for further use. As a check on their purity all cultures were plated, observed, and a subculture of each stock made of a composite of 15 typical colonies from that stock. After this purification the stocks were continued on nutrient dex-

[5] These cultures were obtained through the courtesy of Drs. E. J. Wellhausen, S. S. Ivanoff, C. B. Elliott, and J. H. Muncie.

trose agar. Cultures were transferred once every 4 weeks, allowed to grow at room temperature for 24 hours, and then stored in the refrigerator at about 5° C. Strain characteristics are summarized in table 1. On nutrient dextrose agar the more pathogenic strains form larger, more viscid or mucoid colonies than do the less pathogenic strains.

TABLE 1.—*Growth characters on nutrient dextrose agar of bacterial stocks used for host passage*

Culture No.	Virulence index [1]	Characteristics of 48-hour agar plate colonies				Generation time
		Diameter [2]	Consistency	Surface	Color	
		Microns				*Hours*
S15	36	1,880±120	Dry, granular	Rough	Orange yellow	2.3
D3	45	2,780±520	do	do	Lemon yellow	2.9
A87	48	6,080±1,740	Butyrous	Smooth	do	2.0
FB32	60	4,400±880	do	do	Creamy yellow	2.2
A14	77	11,000±1,520	Mucoid	do	do	1.8
O23	72	6,040±1,400	Butyrous-viscid	do	do	3.2

[1] Virulence index $=100-\frac{\text{Green weight of test plants}\times 100}{\text{Green weight of check plants}}$.

[2] Standard deviation calculated on 300 colony measurements.

Bacteria for inoculum were grown on agar slants for 24 hours, washed off into 5 cc. of nutrient broth, and without further incubation inoculated into test plants. Host plants were inoculated by injecting a small quantity of inoculum into the first nodal region of the plant with a hypodermic syringe. The actual amount of inoculum for each plant consists of the liquid remaining in the needle hole after the syringe needle is withdrawn. The percentage of infection was greater than 99 percent. Reisolations were made 14 to 16 days after inoculation, 8 to 10 plants from each treatment being used. For colony observations, surface colonies were obtained by means of a poured-plate, smear technique.

After reisolation the new passage culture was made up of a large number of colonies (from 50 to 100) picked at random, pooled into one culture, and treated like the stock cultures.

In passage experiments, virulence was determined after two, four, six, and eight passages on a susceptible inbred, GB797. The test plants were grown in greenhouse benches. Not less than 15 plants were inoculated with each culture. Each test culture was replicated three times and compared in virulence with the initial stock culture. After the eighth passage all cultures were given comparative tests against the stock culture and against each passage stock. An analysis of variance (*15*) was made to test the significance of virulence trends observed during host passage.

Green weights of each block of plants were taken 14 days after inoculation. A virulence index was calculated as

$$100-\frac{\text{Green weight of test plants}\times 100}{\text{Green weight of check plants}}.$$

This index may vary from zero to 100, the index increasing as virulence of the bacteria increases. Green weight means the weight of

the top above the first node. Another virulence index has been used and described by McNew (*11*) and Wellhausen (*19*).

The procedure in designating passage cultures has been to list the stock culture, the maize line through which it passed, and the number of passages. Thus S15–GB–8 means the culture obtained by passage of stock culture S15 through maize line GB134 for eight successive passages.

EXPERIMENTAL RESULTS

EFFECT OF SUCCESSIVE HOST PASSAGE ON THE VIRULENCE OF SIX STOCK CULTURES

Subcultures of six stock cultures were passed successively through one resistant and two susceptible lines of maize. Tests for virulence were made after two, four, six, and eight passages. A virulence comparison with the original strain was made at each test, and after eight passages all passage cultures were compared with each other and with the stock strain. The virulence of the stock culture and of the passage strains is given in table 2. There is a pronounced trend for decrease of virulence after passage through the susceptible lines GB134 and WF, and for increase by passage through the resistant line OSF. To such a generalization there is only one minor exception—that of the FB32–WF passage.

TABLE 2.—*Virulence indices of stock and passage cultures during eight successive passages through susceptible and resistant hosts*

Host line	Host passages	Virulence indices of strain [1]—					
		S15	D3	A87	FB32	O23	A14
	Number						
GB134 (susceptible)	0	36	45	48	60	72	[2] 77
	2	27	32	37	48	52	54
	4	19	16	31	50	50	45
	6	21	12	17	33	41	36
	8	18	10	17	27	19	29
Difference [3]		−18	−35	−31	−33	−53	−48
WF (susceptible)	0	36	45	48	60	72	77
	2	39	41	43	64	70	62
	4	22	46	37	71	47	54
	6	25	53	41	68	35	36
	8	19	33	33	65	28	28
Difference [4]		−17	−12	−15	+5	−44	−49
OSF (resistant)	0	36	45	48	60	72	77
	2	45	43	72	54	78	71
	4	58	52	68	63	68	72
	6	60	61	70	72	70	71
	8	56	63	67	65	74	73
Difference [5]		+20	+18	+19	+5	+2	−4

[1] See footnote 1, table 1, and text p. 219.
[2] This is a second series. In the first series the passage culture became completely avirulent apparently because of the culturing of a contaminant.
[3] Differences of less than 20 are not statistically significant.
[4] Differences of less than 23 are not statistically significant.
[5] Differences of less than 18 are not statistically significant.

The S15 and O23 passage series are shown in figures 1 and 2 respectively, where the change in virulence during passage is a progressive trend beginning immediately on passage. In the S15 passage, the ultimate reduction in virulence is brought about within the first four

passages through the susceptible line. Through the resistant line, increase in virulence was most rapid in the early passages but apparently was still rising when the experiment was stopped.

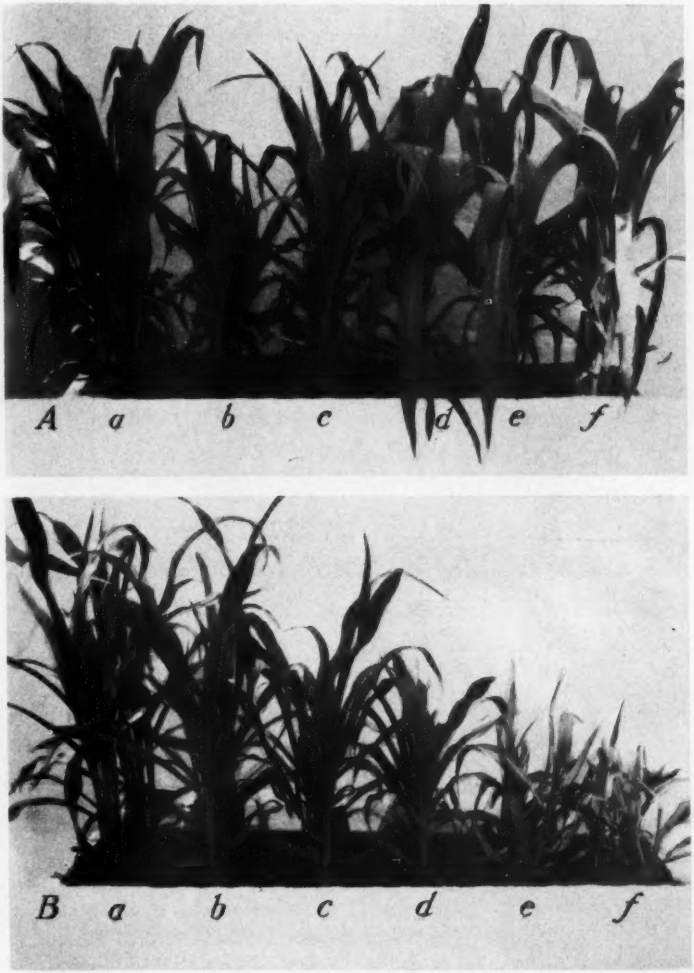

FIGURE 1.—Comparative virulence of S15 stock and passage strains of *Phytomonas stewartii* on a susceptible line of maize 15 days after inoculation: *A*, Successive passage of S15 stock through susceptible host GB134; *B*, successive passage of S15 stock through resistant host OSF. *a*, Check; *b*, inoculated with stock S15; *c*, *d*, *e*, and *f*, respectively, inoculated with S15 passage strains after two, four, six, and eight successive host passages.

Changes in virulence during passage were associated with changes in type of colony growth on nutrient dextrose-agar plates. Loss in

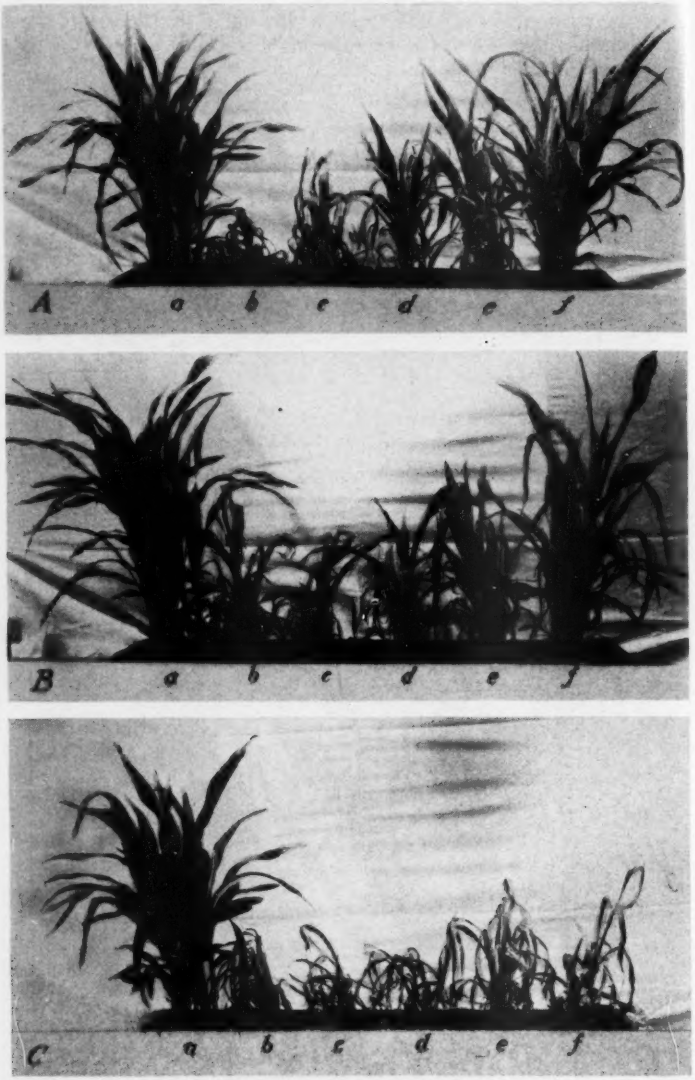

FIGURE 2.—Comparative virulence of O23 stock and passage strains of *Phytomonas stewartii* on a susceptible line of maize 15 days after inoculation: *A*, Successive passage of O23 stock through susceptible host GB134; *B*, successive passage of O23 stock through susceptible host WF; *C*, successive passage of O23 stock through resistant host OSF. *a*, Check; *b*, inoculated with stock O23; *c*, *d*, *e*, and *f*, respectively, inoculated with O23 passage strains after two, four, six, and eight successive host passages.

virulence is associated with a raised, firm colony. Of the four initially smooth cultures passed through the susceptible host—A87, FB32, O23, and A14—in only A14 was the change from smooth to rough phase associated with the decrease in virulence; in the others the colonies remained in the smooth phase, becoming drier, firmer, and more intensely colored. The initially rough S15 and D3 cultures decreased in virulence during susceptible host passage, and remained in the rough phase without apparent colony type changes.

Associated with an increase in virulence by passage through the resistant host, was a more spreading, watery, and viscid growth of the colonies on agar. The change from rough to smooth was observed in passage cultures of both the initially rough stocks, and the increase in the proportion of smooth type was associated with the increase in culture virulence. The highly virulent A14 and O23 cultures remained constant on resistant host passage; A87 and FB32 growth became more watery and spreading as virulence increased.

CHANGE OF VIRULENCE IN PASSAGE STOCKS BY REVERSING HOSTS

To determine whether virulence of a passage culture could be again changed by reversing the host through which it passed, virulent strains that had been passed through resistant OSF for eight times were successively passed through susceptible GB134, and sister avirulent cultures obtained after eight passages through GB134 were passed through OSF. Six successive reverse passages through the new host were made, and virulence determined after two, four, and six passages (table 3).

TABLE 3.—*Change in virulence of passage cultures after the host through which the bacterial stocks were passed had been reversed*

Host line	Host passages	Virulence indices of strain [1]—			
		S15-GB-8	A87-GB-8	O23-GB-8	A14-WF-8
	Number				
OSF (resistant)	0	18	17	19	28
	2	24	36	57	40
	4	41	56	64	47
	6	51	66	70	59
Difference [2]		−33	−49	−51	−31
Host line	**Host passages**	**S15-OSF-8**	**A87-OSF-8**	**O23-OSF-8**	**A14-OSF-8**
	Number				
GB134 (susceptible)	0	56	67	74	73
	2	43	52	50	65
	4	36	42	37	44
	6	28	35	32	35
Difference [3]		+28	+32	+42	+38

[1] See footnote 1, table 1, and text p. 219.
[2] Differences of less than 16 are not statistically significant.
[3] Differences of less than 13 are not statistically significant.

During these reverse passages, changes in colony type and virulence were similar to those described earlier. Virulence of passage stocks

changed as readily and as rapidly as stock cultures that had no host passage.

VIRULENCE VARIATION WITHIN A STOCK CULTURE AND THE EFFECT OF SUCCESSIVE HOST PASSAGE ON VIRULENCE VARIABILITY

As a further test of what changes take place within a bacterial population, a stock culture of intermediate virulence (index 48) was selected to test the virulence variability initially present in the culture as compared with that present after eight passages through susceptible and resistant hosts. Culture A88, selected for this purpose, had been held on agar slants for 3 years with no plating or host passage. One hundred single-cell isolates were obtained by following the plating method described by McNew (*12*). Each colony was plated successively three times, then put on agar slants and held as a stock culture. Virulence tests were made in triplicate.

It is recognized that the use of "single-cell" cultures is commonly limited to cultures derived by the hanging-drop method. McNew (*12*) by direct microscopic examination of poured plates of this species has shown that over 99 percent of the loci occupied by bacteria have only one cell, and after about 11 hours from 80 to 94 percent of these cells have divided, the number depending on the nutrient composition of the agar. Lincoln and Gowen [6] has shown that 4 different strains of *Phytomonas stewartii* are inactivated by X-rays at the same general rate and a rate typical of single particles. As regards the question of whether a colony originates from 2 genetically different strains, Gowen and Lincoln [7] by plating colonies from mixed cultures of white and yellow strains of *Ph. stewartii* found that only 4 colonies out of 2,072 had originated jointly from each of the two strains in the culture. It is further to be stressed, as McNew pointed out, that by making several serial platings and single-colony isolations the probability of obtaining a culture derived from a single cell at least once during this process is tremendously high. The evidence for the plate method of obtaining single-cell colonies or cultures with the species *Ph. stewartii* should be recognized. It is on the basis of this probability and evidence that the term "single cell" is used.

The 100 single-cell cultures from stock A88 varied widely in virulence, most of the colonies tending to possess the same general virulence as that of the parent stock (fig. 3, *a*). Mean virulence of the 100 isolates was 47 with a standard deviation of ± 21. With respect to virulence this stock culture is a population composed of many variants. When such a culture is inoculated into maize, the host is subjected to invasion by a great number of bacterial variants. If certain variants should be better adapted than others to the microenvironment of the host, selective growth would occur, changing the composition of the population. Such has actually been found to be the case, for after 8 passages through the susceptible line the virulence index of the passage culture changed from 48 to 17, with a mean virulence index of 31 ± 16 in the 100 single-cell isolates (fig. 3, *b*). Passage through the susceptible

[6] LINCOLN, R. E. and GOWEN, J. W. INACTIVATION RATE OF BACTERIA BY X-RADIATION IN RELATION TO WAVE LENGTH, TEMPERATURE, AND CELL SIZE. Manuscript.

[7] GOWEN, J. W., and LINCOLN, R. E. A TEST FOR SEXUAL FUSION IN BACTERIA. Manuscript in Genet. Dept., Iowa State College.

host effected a definite shift in the bacterial population toward the avirulent type of organism.

With passage through the resistant line the virulence index of the passage culture changed from 48 to 67, with a mean virulence index of 64 ± 15 in the 100 single-cell isolates (fig. 3, *c*).

Obviously the change in virulence of the passage cultures is due to a shift in the ratio of virulent to less virulent forms initially present. This is contrary to concepts held by certain bacteriological pathologists. For example, Wilson (*21, p. 310*) states: "When a given strain

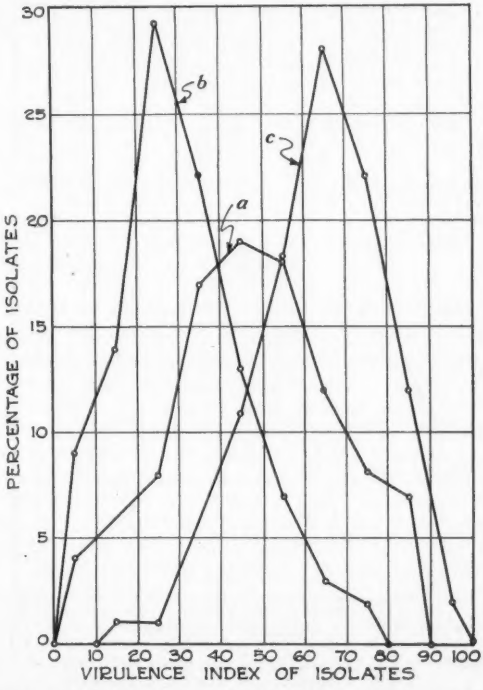

FIGURE 3.—Frequency distribution of pathogenicity of single-cell isolates of *Phytomonas stewartii* from a culture before and after host passage: *a*, Distribution of 100 isolates from initial stock culture; *b*, distribution of 100 isolates from passage stocks after 8 passages through susceptible host; *c*, distribution of 100 isolates from passage stock after 8 passages through resistant host.

comprises organisms showing discontinuous variation in virulence, the virulence of the whole culture is similar to that of the most virulent variants."

To test this hypothesis, inoculating mixtures were prepared that contained different proportions of virulent and avirulent bacteria. The virulence index of these mixtures was tested on susceptible maize and compared with the index of the pure strains. As shown in figure 4, virulence bears a direct relationship to the ratio of virulent to avirulent bacteria. Accordingly, the proportion of virulent and avirulent

types in the inoculum determines the virulence of that culture and not the virulence of the most virulent variant. There is no indication of an "all-or-none" reaction at any dilution in this organism.

HOST PASSAGE OF SINGLE-CELL ISOLATES

Progeny from single-cell stocks should be uniform for all characters if it be assumed that bacteria multiply only by simple fission. In such a culture selection for degree of virulence should be ineffective unless

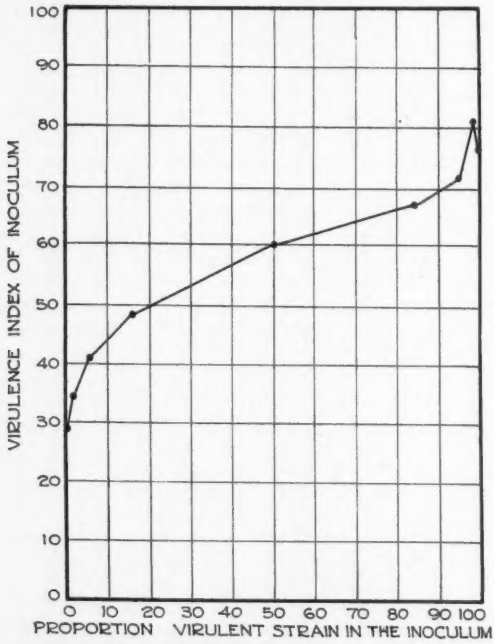

FIGURE 4.--Change in virulence index of inoculum composed of various proportions of virulent and avirulent bacteria.

variation arose. A critical test of whether virulence change is due entirely to selection of existing variation or whether adaptive variation can arise by some other means is furnished by host passage of single-cell cultures. Such a test was made by repeatedly passing virulent single-cell cultures through both resistant and susceptible maize lines and determining the effect of host passage on virulence and colony type.

Single-cell strains from the virulent A14 stock were passed through a susceptible host, but not all cultures were passed through the resistant host since previous work had indicated the lack of any noticeable change during passage of virulent bacteria through a resistant host by the technique of host passage described earlier. Passage was made for six successive times, virulence tests being made after two, four, and six passages (table 4).

TABLE 4.—*Virulence indices and observed variation of single-cell cultures from A 14 stock after six successive host passages*

Culture and stock No.	Initial virulence index [1]	Virulence indices after host passage								Colony variants	
		Susceptible host				Resistant host					
		Passage No.			Difference [2]	Passage No.			Difference [2]	Passage first observed	Virulence indices
		2	4	6		2	4	6			
Stock culture A14	77	66	44	33	−44	75	79	80	+3	2	29
Single-cell cultures:											
502	80	78	51	42	−38					3	13
504	83	81	74	63	−20	79	80	77	−6	3	43
507	73	60	31	56	−17					1	45
508	84	69	66	62	−22	75	76	80	−4	3	48
510	84	80	77	63	−21					5	50
511	82	73	65	54	−28					5	24
512	78	54	57	49	−29	77	73	79	+1	4	42
513	81	83	75	68	−13					3	54
516	85	58	41	24	−61	79	82	74	−11	2	35
517	70	67	57	53	−17					5	45
519	71	68	63	66	−5					None	
520	82	79	65	50	−32	77	75	80	−2	4	39
522	83	74	40	36	−47					2	30

[1] See footnote 1, table 1, and text p. 219.
[2] Differences of less than 15 are not statistically significant.

Perhaps the most striking thing observed with these single-cell culture passages is the consistency with which all cultures that passed through the susceptible line decreased in degree of virulence, while those that passed through the resistant line retained their virulence without appreciable change. Notes on the particular passage in which atypical colony types were first observed are believed to be significant in showing that the atypical colony type was not present at the beginning of the passage, and its origin, from whatever source, appears to be a process occurring rapidly in some cultures, more slowly in others. In only one passage culture were atypical (rough mucoid) colony types observed immediately after the first passage, and in only one culture were atypical colonies not observed during the six passages.

During passage single-cell stocks of A14 shift in virulence and colony type in a manner comparable to the change of the original A14 stock culture. This observation somewhat parallels Hadley's (*3*) general statement on bacterial dissociation. He notes that the more recent work on bacterial dissociation, starting with single-cell cultures, is in no way different from earlier results that involved only colony isolations or in some cases apparently only mass inoculations.

DIFFERENTIAL HOST SELECTION OF VIRULENT AND AVIRULENT BACTERIA

Since certain virulent strains of *Phytomonas stewartii* are characterized by one type of colony and avirulent strains by an entirely distinct type of colony, in a mixture of these two, a morphological character may be used to identify the physiological potentiality (virulence) of a given bacterium. In previous experiments the fact that host constitution plays a definite part in the degree of virulence ultimately attained in the bacterial population has been demonstrated. An experiment was designed to furnish quantitative data on the rate at which change in a bacterial population takes place during host

passage when the initial variance of the population is definitely known and can be followed in time.

A virulent strain of *Phytomonas stewartii*, characterized by large, smooth, spreading, mucoid type colonies, was mixed with an avirulent strain characterized by smaller, slightly rough, raised nonmucoid colonies. The initial proportion of virulent to avirulent colonies was determined on agar plates. Such a mixture was then inoculated into resistant (OSF) and susceptible (GB134) lines of maize. Subsequent proportions of virulent to avirulent types were then ascertained by isolating at suitable intervals of time from the host. For each isolation, lesions from 10 or more plants were macerated together in a small quantity of sterile water, and about 1 cc. of this liquid put into broth and shaken repeatedly for about one-half hour. Dilution plates were then poured from this broth suspension. Surface-smeared plates were used, and only plates that had from 150 to 250 colonies were counted. Counts of all colonies were made with a binocular dissecting microscope at a magnification of 16 ×. Not less than 400 colonies for any isolation, and usually 750 to 1,000 colonies, were counted. Thirteen such experiments were completed, an effort being made to get a wide range in the initial proportion of virulent to avirulent organisms, and to spread the experiments over several months, thus minimizing environmental variations. In terms of percentage of the virulent type of bacteria in the initial inoculum, the range actually obtained was from 15 to 98 percent. Passage time ranged from 14 to 21 days. All experiments were paired, that is, any initial mixed inoculum was always passed through both the resistant and the susceptible lines. In six experiments isolations were made 4 or 5 days after inoculation; in the others isolations were made only at the end of the experiment (tables 5 and 6, respectively).

TABLE 5.—*Progressive change in proportion of virulent type colonies during passage of mixed culture through susceptible and resistant hosts and rate (regression) of change*

Date	Initial proportion of virulent type colonies	Susceptible host—GB134					Resistant host—OSF				
		Proportion of virulent type colonies present indicated number of days after inoculation				Regression	Proportion of virulent type colonies present indicated number of days after inoculation				Regression
		5	9	13	18		5	9	13	18	
	Percent	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*		*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	
Mar. 11, 1938	85	80	77	.....	67	−1.05	85	86	86	89	+0.24
	61	65	60	.....	48	−.76	66	64	71	75	+.82
	24	30	32	26	20	−.23	33	43	45	51	+1.00

Date	Initial proportion of virulent type colonies	Proportion of virulent type colonies present indicated number of days after inoculation				Regression	Proportion of virulent type colonies present indicated number of days after inoculation				Regression
		6	10	15	21		6	10	15	21	
	Percent	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*		*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	
May 22, 1938	98	99	92	82	79	−0.95	96	98	99	99	+0.05
	72	73	67	60	57	−.75	76	80	86	89	+.75
	24	26	30	21	20	−.20	29	34	56	68	+2.20

TABLE 6.—*Change in proportion of virulent type colonies after passage of mixed cultures through the resistant and susceptible host, with average daily rate of change*

Date	Duration of passage	Initial proportion of virulent type colonies	Susceptible host GB134		Resistant host OSF	
			Proportion of virulent type colonies	Average daily rate of change (slope)[1]	Proportion of virulent type colonies	Average daily rate of change (slope)[1]
1938	*Days*	*Percent*	*Percent*		*Percent*	
Mar. 15	14	65	40	−1.96	72	+0.55
Do	14	85	68	−1.33	88	+.22
Apr. 1	19	41	28	−.72	54	+.72
May 8	14	80	63	−1.30	86	+.49
Do	14	28	21	−.52	38	+.73
May 22	20	65	49	−1.45	79	+.74
Aug. 5	15	15	8	−.50	37	+1.49

[1] Figures in this column represent m in the formula for a straight line between 2 points, $m=\frac{y_1-y_0}{x_1-x_0}$

Strains of A14 and S15 were used for most of the mixed culture work. These were particularly favorable because they represented extremes

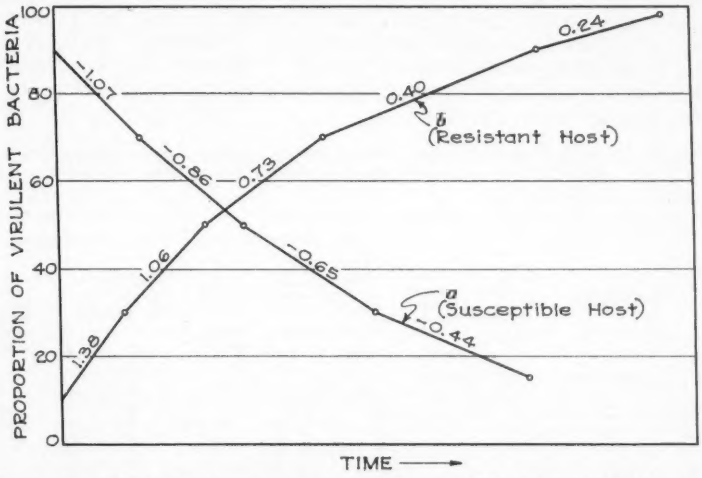

FIGURE 5.—Effect of host passage on proportion of virulent to avirulent bacteria in mixed culture inoculations: *a*, Susceptible host; *b*, resistant host. Figures denote regression coefficients in short experiments.

of virulence, were very stable in culture, and were distinct in colony morphology.

There is a highly significant positive correlation of $r=0.95$ between the ratio of virulent and avirulent bacteria in the inoculating suspension and that obtained by isolating from the first definite lesion on the leaf, showing that bacteria that actually grow and produce a diseased condition are reliably estimated by plating directly from the inoculating medium.

As shown earlier (*9*), upon passage of the mixed cultures through the susceptible host there is a differential selection favoring the avirulent type of bacteria. Figure 5, *a*, shows this change in an inoculum with

an initial proportion of 90 virulent to 10 avirulent bacteria decreasing during passage through the susceptible host to 15 virulent and 85 avirulent bacteria. The whole sweep of this reaction is curvilinear, apparently asymptotic to the replacement of the virulent type by the avirulent. Because of the early death of the susceptible host the full reaction is not expressed in any one experiment, but this curve is built up from the short experiments given in tables 5 and 6, in which the initial proportion of virulent to avirulent bacteria varied between 15 and 98 percent of the virulent type.

When passage is made through the resistant host there is a differential selection for the virulent type. Figure 5, *b*, shows the increase in the proportion of virulent bacteria during passage through the resistant host. Curves *a* and *b* were determined in a comparable manner, differing only in direction of change.

To test the conclusion that rate and direction of selection are dependent upon host resistance, the same technique and bacterial stocks were used in experiments in which 14 new maize lines with different degrees of resistance were employed as host. Host resistance as rated by Wellhausen (*18*) ranges from 1 to 5. One represents a degree of resistance in which there is no noticeable stunting of the inoculated plants and 5 represents extreme susceptibility, plants dying in 10 to 20 days after inoculation with a virulent strain of bacteria. Ratings 2, 3, and 4 are intermediate stages. Data on change in the proportion of virulent bacteria after passage, together with the rated resistance of each line, are given in table 7.

TABLE 7.—*Change in proportion of virulent type colonies after passage through various maize lines of known resistance to Phytomonas stewartii, with average daily rate of change*

Maize line or cross	Resistance of line [1]	Test 1, 19-day passage			Test 2, 21-day passage		
		Proportion of virulent type colonies present		Average daily rate of change (slope) [2]	Proportion of virulent type colonies present		Average daily rate of change (slope) [2]
		Initial	Final		Initial	Final	
		Percent	*Percent*		*Percent*	*Percent*	
GB134	5	41	28	−0.7	65	49	−0.8
887	5	41	24	−.9	65	47	−.9
Sto	5	41	33	−.4	65	31	−1.6
WF	5	41	23	−1.0	65	45	−.9
G540	4	41	30	−.6	65	56	−.4
Mc	4				65	60	−.2
1339	3	41	36	−.3	65	60	−.2
Idt	3				65	63	−.1
C1	2	41	43	+.1	65	68	+.1
BM	2	41	19	−1.2	65	33	−1.5
PR	1	41	56	+.8	65	71	+.3
MK	1	41	52	+.6	65	83	+.9
TR	1	41	63	+1.2			
OSF	1	41	51	+.7	65	79	+.7
MK × OSF	1				65	91	+1.2
OSF × GB134	1				65	79	+.7

[1] Resistance as indicated: 1, Highly resistant; 2, resistant; 3, intermediate; 4, susceptible; 5, highly susceptible. (From Wellhausen (*18*).)

[2] See footnote 1, table 6.

It is again apparent that direction and rate of change in proportion of virulent bacteria during passage are dependent upon host resistance. There is a high correlation between host resistance and rate and direction of change in the bacterial population. The direction of

change in all lines of intermediate to low resistance (susceptible)—rates three, four, and five—is toward a lower proportion of virulent organisms. In resistant lines (rating one or two) the direction is toward a higher proportion of virulent organisms. One exception to this is noted—that of BM, a resistant early sweet corn inbred from the Black Mexican variety. This resistant line gave a selective reaction similar to that of a highly susceptible line. An explanation is not available at present

Another test of host selection is to vary the virulence of the bacteria in a mixed culture used to judge selection rate and direction. Each of 7 bacterial stocks with virulence index ranging from 14 to 80 was mixed with a common avirulent stock (S15), passed through the same hosts (GB134 and OSF), and the direction and rate of change in the bacterial mixture determined.

All bacterial stocks were chosen both for a colony type and virulence that were very different from S15 stock. Of the stocks used, S15 and A14 have already been described. RM is a rough mucoid variant observed to arise from A14 during passage through the susceptible line. All other stocks were selections from the A88 experiment, the summarized data of which were presented in an earlier section. The technique of mixed culture selection is the same as that described earlier. S15 stock was used as the standard by which selection was judged, that is, the common stock with which all other strains were mixed. Results are presented in table 8. The degree of virulence was determined on GB797.

TABLE 8.—*Change in proportion of S15 type bacteria during 16-day passage through susceptible and resistant host and average daily rate of change*

Stock mixed with S15	Virulence index [1] of pure culture	Initial proportion of S15 bacteria	Susceptible host, GB134		Resistance host, OSF	
			Proportion of S15	Average daily rate of change (slope) [2]	Proportion of S15	Average daily rate of change (slope) [2]
		Percent	*Percent*		*Percent*	
S15	30					
11	80	54	68	+0.9	39	−1.0
A14	76	57	68	+ .7	41	−1.1
93	63	50	59	+ .6	38	− .8
83	56	45	50	+ .3	40	− .3
RM	48	62	68	+ .4	57	− .3
47	37	42	44	+ .1	40	− .1
16	14	48	45	− .2	53	+ .3

[1] See footnote 1, table 1, and text p. 219.
[2] See footnote 1, table 6.

Direction and rate of change in the S15 type organism when passed through a common host (OSF or GB134) are both dependent upon the virulence of the bacterial strain with which it is mixed. The more extreme the virulence of the variant with which S15 is mixed, the more rapid the shift in the bacterial population. On passage through the susceptible line, selection was in favor of S15 when the variant with which it was mixed was more virulent; against S15 when the second strain was less virulent. In the resistant host, OSF, the converse was true. The experiment was not repeated since the results

are consistent and entirely comparable with the more extended S15–A14 mixtures.

These experiments, in which host resistance and bacterial virulence were varied, show that during host passage the direction and the rate of change of virulent and avirulent bacterial types in a population are dependent upon both host resistance and bacterial virulence. Where either resistance or virulence is held constant such a change is a direct function of the other; where both may vary, as would be be true in a natural population, the change is due to an interaction of both variables.

NATURAL RATE OF MUTATION IN PHYTOMONAS STEWARTII

In preceding sections data have been presented showing that by host passage a change in virulence of bacterial populations is brought about, the direction and rate of this change being dependent upon host resistance and bacterial virulence. Where the initial inoculum originated from several colonies it has been shown that such cultures are composed of bacterial variants with different degrees of virulence (A88 experiment). For such cases selection explains the known facts of virulence change. When single-cell cultures are used (as was done with A14 stocks) some origin for the variability upon which selection works must be postulated. Since mutation is one known method for emergence of variability, it is important to ascertain whether mutations actually occur, their frequency, and the nature of such mutations.

All strains of *Phytomonas stewartii* used for mutation work were purified genetically by repeated single-celling. Agar colonies from such cultures were extremely uniform as to color, size, shape, and surface character. Bacteria from a single colony were used to inoculate 10 cc. of nutrient broth, a single, different colony being used for each tube of broth. These tubes were incubated at 27° C. for 18 to 20 hours, shaken repeatedly, diluted, and plated. Plates were incubated at room temperature for 3 days, then examined under a binocular dissecting microscope at 16 diameters. The total number of colonies was estimated by counting one-tenth of the area of a representative number of plates poured from each tube. Each observed variant was put into broth, replated, and the variant characters rechecked on this subplate before it was considered that a mutation had occurred.

For calculation of the maximum mutation rate the total number of colonies under observation was divided by the number of mutant colonies observed. This is considered a reasonable procedure since after a mutation arose, that mutant would increase in some ratio to the parent stock, the actual number of mutants observed depending upon the cell generation in which the mutation had originated and whether the mutation had originated in more than one cell. For the minimum mutation rate it has been assumed that a given mutation arose only once in a given tube, and repeated observations of that mutant merely indicate its later growth. For interpretation of these data it must be remembered that most variables tend to keep the mutation rate at a minimum.

Stock S15 and two color variants derived from this stock have been used to determine the mutation rate of characters distinguishable

from the normal strain by colony type and color. This stock was chosen because it was characterized by rough, raised, dark-yellow, small colonies that remained distinct and individual even when the colonies were crowded on agar plates. The small colonies of this strain allowed many more colonies per plate than any of the larger spreading types. Stock 101 is a pale-yellow derivative from S15, appearing to vary from the parent only in color. Stock 105 is a white derivative from S15.

TABLE 9.—*Natural mutation rate of three strains of Phytomonas stewartii after 18 hours' growth in nutrient broth* [1]

Bacterial strain, number of tubes containing mutants, and rate of mutation	Tubes treated as units	Total colonies observed	Mutant colonies observed of type indicated							
			Dark yellow	Pale yellow	White	R2	R3	R4	Smooth	Sectored
	Number	*Thousands*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
S15 (dark yellow, rough)	15	836	------	28	8	4	1	------	41	------
Number of tubes in which mutation was observed	------	------	------	13	6	2	1	------	11	------
Maximum rate of mutation per million individuals	------	------	------	33.3	9.6	4.8	1.2	------	49.0	------
Minimum rate of mutation per million individuals	------	------	------	15.5	7.2	2.4	1 2	------	13.2	------
101 (pale yellow, rough)	20	1,645	160	------	281	13	------	3	15	8
Number of tubes in which mutation was observed	------	------	17	------	5	11	------	2	9	8
Maximum rate of mutation per million individuals	------	------	97.3	------	170.8	7.9	------	1.8	9.1	4.9
Minimum rate of mutation per million individuals	------	------	10.3	------	3.0	6.6	------	1.2	5.5	4.9
105 (white, rough)	10	620	------	------	------	1	------	------	------	------
Number of tubes in which mutation was observed	------	------	------	------	------	1	------	------	------	------
Maximum rate of mutation per million individuals	------	------	------	------	------	1.6	------	------	------	------
Minimum rate of mutation per million individuals	------	------	------	------	------	1.6	------	------	------	------

[1] Complete tables in unpublished doctorate thesis. LINCOLN, R. E. RESISTANCE TO BACTERIAL WILT PATHOGEN OF MAIZE AND GENETIC HOST-PARASITE INTERACTIONS. 1939.

Mutation rates in the three stocks are given in table 9. Perhaps the most striking observation is the difference in mutation rates of the three stocks, the rate in the white stock being much lower than in the yellow stocks. Within the yellow stocks, usually the same characters were observed to vary, yet each stock yielded a type of variability not found in the other stock. The color mutations are interesting in showing that there are different intensities of yellow and that certain characters are more stable than others. Thus the mutation dark yellow to pale yellow and the reverse mutation—pale yellow to dark yellow—occur about equally often, whereas dark yellow mutates to white more readily than light yellow mutates to white. White is extremely stable, the mutation of white to yellow never having been observed. An examination of the different "rough" colonies shows that there are no fewer than four distinct and different types. These rough types have been designated as R1, R2, R3, and R4; R1 being the parental type in all three stocks used. The mutants classified as "smooth" probably are really several different smooth-type mutations rather than the same mutation in all cases. Because of the relation of smoothness to virulence, these smooth mutations are particularly significant.

The mutations are apparently stable from the time of origin. A pure culture of the variant type can be obtained directly from the variant colony, no type of segregation having been observed. Mutations arising as sectored colonies are apparently the same as mutations observed as entire colonies. Mutation in one character does not cause a mass mutation, for only one character has been observed to mutate at a time. Thus a colony having a color mutation is not morphologically different from the parent stock. This fact and the occurrence of reverse mutation suggests that the mutations observed are not "loss" type mutations such as would be occasioned by a chromosome deficiency. These variants may be point mutations in the genetic sense. Their mutation rates of 1 to 20,000 to 800,000 are not different from the known rate of many genes of higher organisms.

The term "mutation" rather than dissociation has been used because the observed variations are stable, hereditable, and transmissible from parent to daughter cell. Such variant characters are stable under a variety of environments. The change from dark-yellow to pale-yellow colony color is one differing at least in quantity of pigment while the white form lacks all carotenoid pigment (unpublished data). Experimental evidence for mutation is furnished by Lincoln and Gowen,[8] who obtained all the variants observed in this work as well as additional variation after treatment of this stock with X radiation. Rate of mutation after treatment with X radiation was much higher than the natural mutation rate given in this paper.

Since it was of particular interest to the interpretation of virulence change by host passage, several mutations observed to arise in pedigree cultures were tested for virulence. This was the only physiological test made of these variants. A single, unreplicated test was made. The virulence of each variant was compared with that of the type stock from which it arose (table 10).

TABLE 10.—*Virulence indices of mutants from R1 type stock*

Source and culture No.	Variant type	Virulence index [1]	Source and culture No.	Variant type	Virulence index [1]
From S15 stock (dark yellow):			From 101 stock (pale yellow):		
Parental type	R1	29	Parental type	R1	31
456	Smooth	48	409	Smooth	17
459	do	43	446	do	48
461	do	53	438	R2	25
462	do	36	414	R4	46
466	do	13	From 105 stock (white):		
473	do	47	Parental stock	R1	45
487	do	56	422	R2	34
454	White R1	49	480	R3	17
455	do	55			
401	Pale yellow R1	51			
407	do	31			
474	R2	36			

[1] See footnote 1, table 1, and text p. 219.

Stocks from which these mutations arose were all rough type (like S15). All mutations except those marked smooth are still in the rough phase. The increase in virulence discovered in most of the smooth mutations is important and is consonant with results in pas-

[8] LINCOLN, R. E., and GOWEN, J. W. PRODUCTION AND RATE OF MUTATION IN PHYTOMONAS STEWARTII BY X RADIATION. Manuscript.

sage cultures. The exceptions need further study. It seems possible for virulence to be high or low in either the rough or smooth phase.

The demonstration of mutations in *Phytomonas stewartii* furnishes a source of variability great enough to account for the variation in virulence and colony type observed after host passage. This is particularly important in accounting for changes observed in single-cell cultures during host passage.

EXPERIMENTS FOR TESTING SEXUAL FUSION IN PHYTOMONAS STEWARTII

If bacteria have sexual fusion then recombination and segregation of characters would be possible. This question is fundamental to an interpretation of the virulence changes in this investigation. Gowen and Lincoln [9] have presented data on a technique which furnishes a certain type of information on this point. They grew in mixed broth cultures pure white and yellow strains of *Phytomonas stewartii*, plated from these mixed cultures at intervals, and replated from single colonies to observe possible segregation. If fusion and segregation were common, some of the colonies should segregate for both yellow and white colonies. There was no evidence of such segregation from their data. A possible limitation of their work is that only three repeatedly single-celled yellow cultures were tested against a white culture. If bacteria were of + and − strains the probability of testing unlike strains by this method would not be very high. Environmental conditions may also play an important part in reproductive processes. To correct for these two possible limiting factors and to collect more data by this technique, seven cultures that had been carried in stock without host passage or single celling were mixed with the white stock and passed through a resistant and a susceptible host. After 20 days' growth in the host, isolations were made and agar colonies plated (table 11).

TABLE 11.—*Colonies after 20 days' host passage of mixed cultures of white and yellow bacteria plated to test for sexual fusion of white and yellow cells*

Yellow strain with which white S15 variant was mixed	Single colonies plated				Plates showing yellow and white colonies from—	
	Susceptible host		Resistant host		Yellow	White
	Yellow	White	Yellow	White		
	Number	*Number*	*Number*	*Number*	*Number*	*Number*
A14	135	22	164	27	2	0
O23	218	25	160	15	2	0
A87	100	5	112	5	0	0
S15	157	18	103	12	1	0
FB32	120	10	84	10	4	0
D3	62	0	49	0	0	0
Total	792	80	672	69	9	0

A few yellow colonies showed segregation for white. Further plating from these yellow colonies did not show any segregation. This would be expected if the original colony started from two bacteria, one white, the other yellow. There is no indication of sexual fusion that may be determined by this method with any of the stocks used.

[9] See footnote 7.

Such being the case, it seems unimportant whether a culture is derived from a single cell at the time the culture is taken or whether the culture is made from a group of cells one or two generations removed from a common cell since variation would be limited to such processes as mutation or somatic segregation.

DISCUSSION AND CONCLUSIONS

The opinion prevalent among bacteriologists is that host passage maintains or increases bacterial virulence. Recently Wellhausen (*19*) showed that the genetic constitution of the host is important in determining whether bacterial virulence is enhanced, maintained, or decreased.

This was verified in the present experiments by passing several stocks of *Phytomonas stewartii* through numerous lines of maize. Six stocks of bacteria were successively passed through inbred resistant or susceptible hosts. The results, with minor exceptions, prove that successive passage through susceptible lines decreases virulence, while passage through resistant lines increases the virulence of bacterial stocks.

More important, the virulence of 13 single-celled virulent stocks was decreased by passage through the susceptible host. Passage of these stocks through the resistant host maintained their virulence. The virulence of single-cell cultures changes in a manner comparable to that of stock cultures during host passage, although at a slower rate.

Virulence changes in mass cultures may be due entirely to host selection of variations known to be present. Quantitative proof of such differential host selection was obtained by inoculating susceptible and resistant hosts with mixtures of virulent and avirulent bacteria of known proportions, and at suitable intervals determining the direction and rate of change in the proportion of types. It is concluded that direction and intensity of selection for virulence change is largely dependent upon the resistance or susceptibility of the host.

Host passage of 13 single-cell cultures furnishes critical proof that adaptive variation does arise by some means and is subsequently selected.

The mutation rate for colony morphology and color was determined in nutrient broth on three bacterial strains. The mutation rate of characters studied was entirely comparable with mutation rates of genes determined experimentally in higher forms. Since evidence for sexual fusion in this species is entirely negative, mutation is probably the chief, perhaps the only, source of variation

Mutation and natural selection within the host have been shown to be two important agencies in bringing about observed changes in bacterial virulence during host passage. In this respect the mechanisms for the evolution of bacterial virulence are not greatly different from those in higher organisms.

Considering the large number of individuals and generations possible in a few days of bacterial growth, combined with a mutation rate comparable to that in higher forms, it is entirely possible that selection processes in the microenvironment of the host are capable of effecting the observed changes in virulence of this pathogen.

Virulent bacteria kill the susceptible host in 10 to 15 days but only stunt the resistant host; avirulent bacteria stunt the susceptible host,

but in the resistant host soon become limited to a few early lesions. Successive passage of a bacterial stock through either the resistant or susceptible host effects a change in virulence of the bacteria to a point where that host is stunted noticeably but not killed. The host does not eliminate the parasite by outgrowing it. Once this virulence condition is reached, further host passage does not cause an appreciable change in virulence. Over long periods of time selection within the microenvironment of the host would be toward such a point of equilibrium.

SUMMARY

Six stock cultures of *Phytomonas stewartii* were successively passed through resistant and susceptible inbred lines of maize. Passage through the susceptible host decreased virulence, while passage through the resistant host increased virulence.

Change in virulence during passage was correlated with changes in colony growth on agar plates. Loss in virulence was always associated with a raised, firm type of colony but not necessarily with a change from the smooth to the rough phase. With an increase in virulence the colonies became more spreading, watery, and viscid. The change from rough to smooth and from smooth to rough was observed during passage.

Virulence of passage stocks could again be changed by reversing the host through which these stocks were passed.

Single-cell colonies from an old stock culture show that it is composed of many variants differing in virulence. Successive passage of this stock culture through susceptible maize decreased the proportion of virulent variants, while passage through resistant maize increased the proportion of virulent variants.

Virulence of a culture is a direct function of the proportion of virulent and avirulent organisms present in the culture.

As in stock cultures, virulent single-cell cultures lose virulence by passage through the susceptible host, while passage through the resistant host maintains high virulence.

Mixtures of virulent and avirulent bacteria of known proportions were inoculated into susceptible and resistant hosts, and the change in the proportions of bacteria followed at given intervals of time. There was differential selection for the avirulent type in the susceptible host and for the virulent type in the resistant host. With 1 exception this was true for 14 lines and 2 crosses of maize. Both rate and direction of change are functions of host resistance and bacterial virulence.

After 18 hours' growth in nutrient broth the mutation rate of colony color and colony morphology was determined on three bacterial strains. The mutation rate of these characters is of the same order as the known mutation rate of the genes in higher forms. The calculated mutation rate ranged from 1 in 20,000 to 1 in 800,000 individual cells.

The virulence of the mutations varied greatly from the virulence of the parent stock. Both increases and decreases in virulence were noted.

Sexual fusion of white and yellow strains of *Phytomonas stewartii* in the living host could not be demonstrated experimentally.

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RELATION OF THE NEAR-WILT FUNGUS TO THE PEA PLANT [1]

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INTRODUCTION

Near-wilt of pea (*Pisum sativum* L.) was first described by Snyder (*5*).[3] Later Snyder and Walker (*7*) defined the causal organism of the disease as *Fusarium oxysporum* Schlect. f. 8 Snyder. Snyder (*6*, p. 458) also reported that *F. vasinfectum* Atk. var. *pisi* Van Hall, one of the organisms involved in the complex St. John's disease in Europe, is synonymous with *F. oxysporum* f. 8. Appel (*1*), many years before Snyder described near-wilt in the United States, found that germination was reduced when healthy seed of field peas was inoculated with a spore suspension of *F. vasinfectum* var. *pisi*. Snyder and Walker (*7*) reported that small rootlets of near-wilt-infected pea plants may show evidence of decay, and in some cases may be rotted away, but that in general the root and foot of the plants are free of any conspicious decay of the cortex. There may be, however, conditions of infection which favor invasion of the cortex, and symptoms of root rot attended by a slight discoloration of the cortical tissues may be detected. Virgin and Walker (*10*) found that there was a distinct coincidence between the appearance of near-wilt and the beginning of the blossoming period. Starr (8), working in Minnesota with a *Fusarium* wilt of pea, found the causal organism to be seed-borne and the fungus to be present in the seed coats but not in the cotyledons.

The purpose of the investigation reported in this paper was to study the mode of entrance of the near-wilt fungus into the host, the course of the pathogen after penetration, its effect on the host, and the possibility of the organism being seed-borne.

MATERIALS AND METHODS

The following varieties of peas were used in this study: Wisconsin Perfection, Wisconsin Early Sweet, Dwarf White Sugar, Alaska, Prince of Wales, Penin, Senator, all very susceptible to near-wilt, and Rogers K, a variety possessing decided resistance to the disease. The fungus used throughout the investigation was from a culture isolated by the writers and identified as *Fusarium oxysporum* f. 8 by W. C. Snyder of the University of California.

Peas were germinated aseptically; and when the radicles were about an inch long, they were placed in contact with the near-wilt fungus which was growing on potato-dextrose agar. These peas were left

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[2] Appreciation is expressed to Wilbur Schroeder, formerly a fellow in Plant Pathology, University of Idaho, for his assistance on certain parts of this investigation.

[3] Italic numbers in parentheses refer to Literature Cited, p. 248.

for different lengths of time before being fixed and sectioned. Other pea radicles were placed in contact with the fungus for various lengths of time and then transferred to a moist chamber and suspended on a mesh screen. At suitable intervals they were removed, placed in a fixative, and were later sectioned. Peas were planted in sterilized sand which had previously been infested with the near-wilt fungus, and when the seedlings were 5 to 8 days old, they were removed and plated out on acidified potato-dextrose agar in order to determine the location of the fungus. Stems from plants that were dying from the disease were also fixed and sectioned.

Seeds of the variety Wisconsin Early Sweet which had been surface-sterilized were planted in sterilized sand, but before they were covered they were sprayed with a heavy suspension of macroconidia of the near-wilt fungus. After 7 days the affected seedlings were removed, washed thoroughly, and plated out on potato-dextrose agar without being surface-sterilized.

The fixative used, consisting of two solutions, A and B, was a combination of Bouin's fluid and Karpechenko's modification of Navashin's solution. Solution A, containing 100 cc. of water, 1 gm. of chromic acid, and 4 cc. of glacial acetic acid, was saturated with picric acid. Solution B consisted of formalin. Two parts of solution A were mixed with 1 part of solution B just before using.

The process of dehydration and infiltration with paraffin was carried out according to the schedule given by Rawlins (*3, p. 21*), in which cedar oil was used following a series of alcohols of various concentrations up to 95 percent. For mature pea stems, which were quite woody, butyl alcohol was substituted for ethyl alcohol after the material had been brought to 70-percent concentration. Sections were cut 8 to 15 μ in thickness. The material was stained with Delafield's haematoxylin followed by orange G in 95-percent alcohol.

For seed-transmission studies, peas were grown in near-wilt-infested soil. The vines were supported on trellises to prevent the pods from coming in contact with the soil, and seed was harvested from plants that survived. Seed was also collected from diseased plants grown on naturally infested soil. Both lots of seed were planted in soil known to be free from the near-wilt fungus. For isolation of the organism from the seed the latter was sterilized by placing in 70 percent alcohol for 30 seconds and then in a solution of sodium hypochlorite (B. K. 1–10) for 10 minutes. The seeds were then soaked in sterile water for 4 hours to facilitate separating the seed coat from the cotyledons, and the various parts were then placed on plates of potato-dextrose agar.

EXPERIMENTAL RESULTS

PENETRATION

The near-wilt fungus was found to penetrate the young pea seedling most commonly at the root tip and the cotyledonary node. Penetration occasionally took place at various points along the root and epicotyl and sometimes at the growing point of the latter. No noticeable differences were observed in the penetration of the very susceptible variety, Wisconsin Perfection, and the resistant variety, Rogers K.

Repeated platings of 5- to 8-day-old seedlings grown in near-wilt-infested sand showed the location of the fungus at the cotyledonary node and the root tip. In order to make sure that the fungus did not enter at other points and when plated out on agar grow out at the cotyledonary node, some of the seedlings were cut in small pieces and plated. Many of the seedlings showed infection only at the cotyledonary node (fig. 1, *A*), others at the root tip, and some at both places (fig. 1, *B*). Occasionally in some of the seedlings plated, the fungus was found at the tip of the epicotyl (fig. 1, *C*), and in others it was found to grow out along the epicotyl a short distance above the cotyledonary node (fig. 1, *D*).

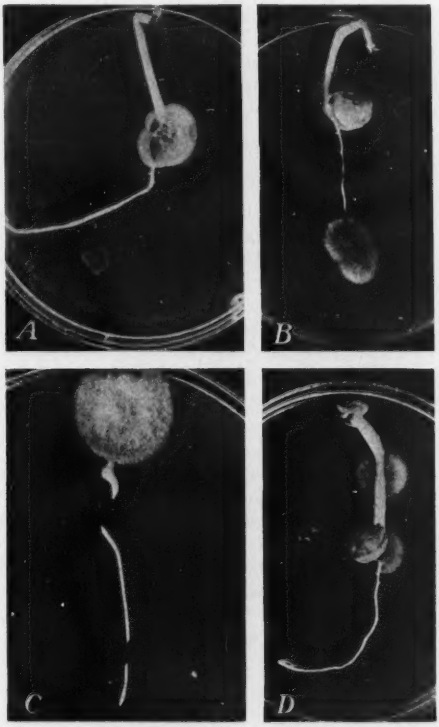


FIGURE 1.—Platings of seedlings grown in near-wilt-infested sand: *A*, 5-day-old Rogers K seedling showing location of organism at the cotyledonary node; *B*, a similar seedling showing location of infection at both the root tip and the cotyledonary node; *C*, 5-day-old Wisconsin Perfection seedling showing location of fungus at the growing point of the epicotyl; *D*, a similar seedling showing location of infection in the epicotyl and at the cotyledonary node.

The fungus accumulated in a mass on the root-cap cells (pl. 1, *A*), where the hyphae penetrated between the cells and soon entered the embryonic region. Intercellular preceded intracellular penetration in this region, but the reverse order frequently occurred in older tissues. After the organism had gained entrance into the meristematic region, it progressed with little difficulty through the region of elongation into the xylem vessels of the young root. Very often the entire root tip was so thoroughly invaded that it was impossible to distinguish the host tissue from the fungus mass.

The near-wilt fungus was able to penetrate the root at points other than the root tip and cotyledonary node. When a mass of mycelium accumulated at some point along the root, the hyphal threads soon penetrated the epidermis and cortex both intercellularly and intracellularly (pl. 1, *B*). The fungus progressed up and down the cortex as it made its way inward to

the stele (pl. 1, *C*), where it entered the xylem elements by way of the pits.

Seedlings resulting from the seed that was sprayed with a suspension of macroconidia were rotted at the cotyledonary region and for some distance below it. The near-wilt fungus grew out abundantly from the rotted area, indicating that the organism had thoroughly penetrated the cortex. No other organisms were present.

Infection in lateral roots took place similarly to that in primary roots. Penetration was not observed where the emerging secondary root ruptured the cortex and epidermis.

SUBSEQUENT DEVELOPMENT OF THE FUNGUS AND THE EFFECT ON ITS HOST

After initial penetration the organism soon made its way both intercellularly and intracellularly to the stele of the root. Some development of the fungus occurred in the cortex, occasionally causing complete disintegration and a consequent seedling rot. Upon entering the stele the organism was confined there until the advanced stage of the disease developed. The fungus was found in both large and small xylem vessels, and it was through these that the organism made its upward advance from the point of infection (pl. 1, *D* and *E*). Progress from one vessel to another took place through the pits. Many of the xylem vessels became completely plugged with hyphae, and microconidia were frequently observed in some of the larger vessels.

The fungus was found to progress the entire length of the stems in many of the susceptible varieties of peas. When stems from wilted plants were placed in moist chambers for 60 hours, the fungus grew out profusely from them (fig. 2). A number of stems were cut and separated at each internode to make sure that no upward growth of the organism occurred while in the moist chamber. However, the fungus was still found to occur throughout the length of many of the stems, indicating that the fungus had already advanced through them before being placed in the moist chamber.

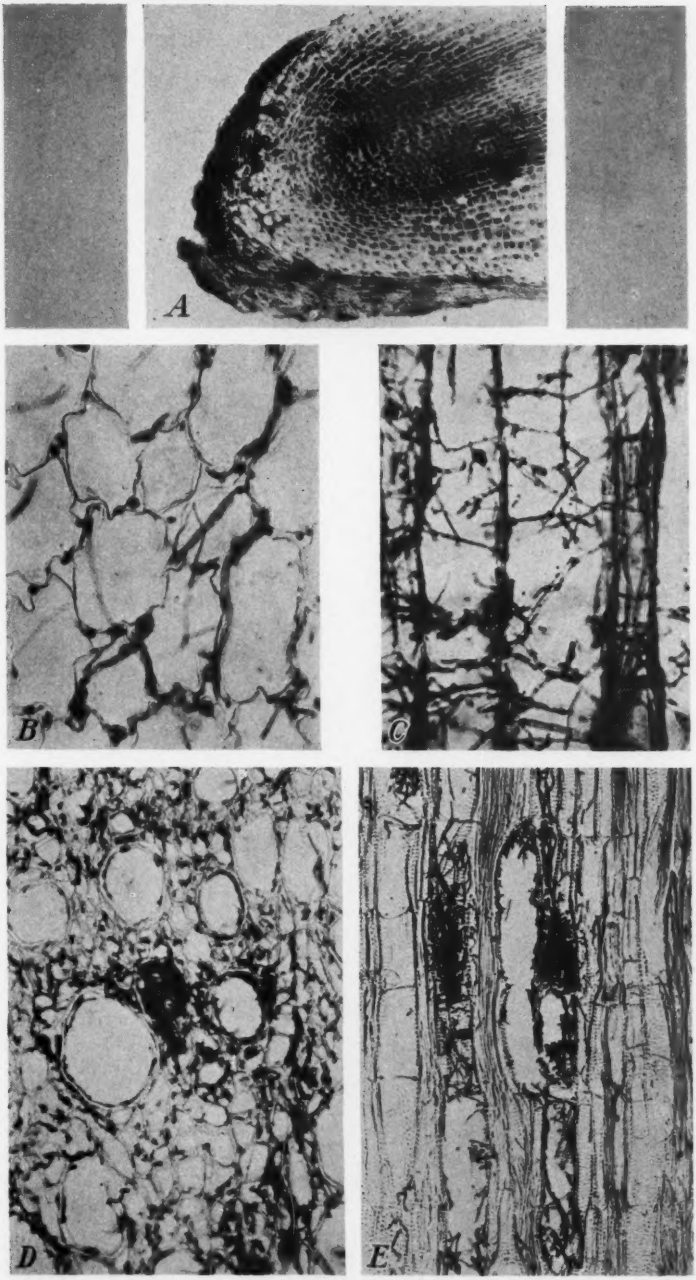
In the case of resistant Rogers K and some promising hybrids of which it was one of the parents, the fungus did not advance beyond the eighth node when grown on near-wilt-infested soil.

SEED TRANSMISSION

Since the fungus was found to advance from the root through the entire length of the stem of susceptible pea varieties, and because of the fact that the disease does not ordinarily kill the plant until the middle of its development or later, usually when pods are setting, it seemed highly probable that the fungus might reach the seed through the vascular system.

EXPLANATORY LEGEND FOR PLATE 1

A, Longitudinal section of an infected root tip of a Rogers K pea plant, showing accumulation of mycelium around it and intercellular penetration; *B*, cross section of cortex of seedling root, showing fungus in both intercellular and intracellular relation to the host cells; *C*, longitudinal section of cortex of seedling root, showing a considerable amount of the fungus present, infection having taken place along the surface of the root; *D*, cross section through the first internode of a plant that died at the time it was setting pods; *E*, longitudinal section through the first internode of the same plant. Note abundance of mycelium in certain of the vessels.



FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.

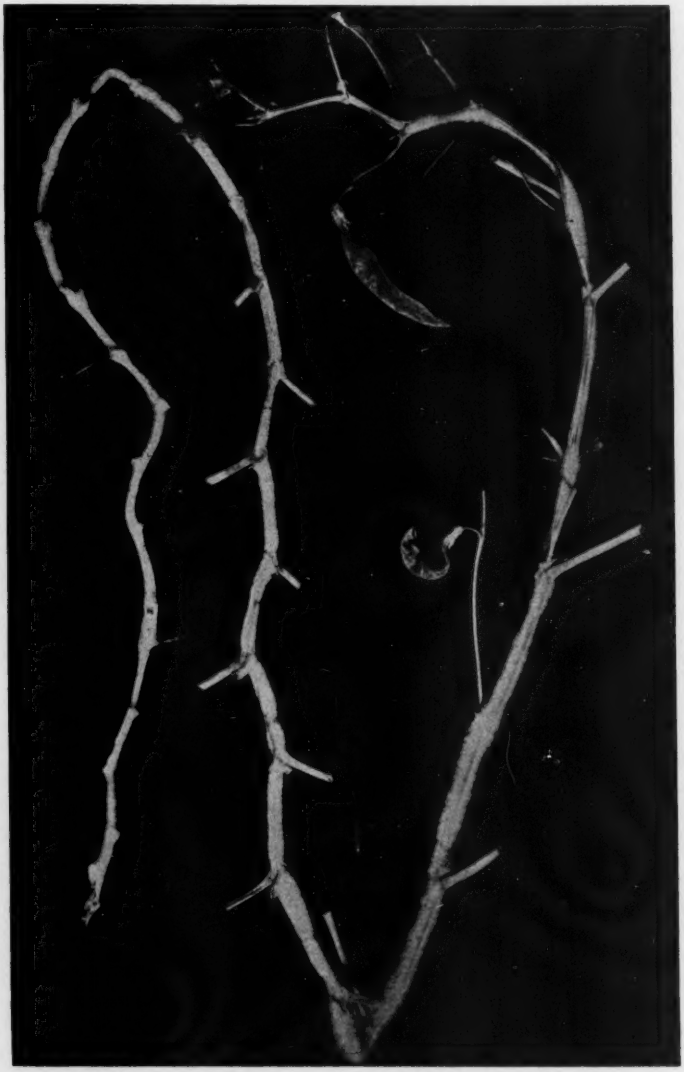


FIGURE 2.—Near-wilt-diseased vine of Dwarf White Sugar placed in a moist chamber for 60 hours in order to promote fruiting of the pathogen on the surface as an indication of the extent of its invasion. The fungus extended almost to the tip of the stem which was 3 feet 4 inches in length.

Seeds of several varieties of peas were collected from near-wilt-diseased plants grown on naturally infested soil in the field. These plants generally had set one or two pods, although many of the pods failed to develop seed. The seed coats were separated from the cotyledons of a number of these seeds when plated out on potato dextrose agar. The near-wilt fungus was found in the seed coats and in the cotyledons, but not necessarily in both in the same seed (fig. 3). The results of the isolation studies are shown in table 1. Several isolates were also obtained from seed produced by susceptible plants grown on near-wilt-infested soil in the greenhouse.

Seed collected from two varieties of peas, Wisconsin Perfection and Prince of Wales, grown on a naturally infested field in Wisconsin

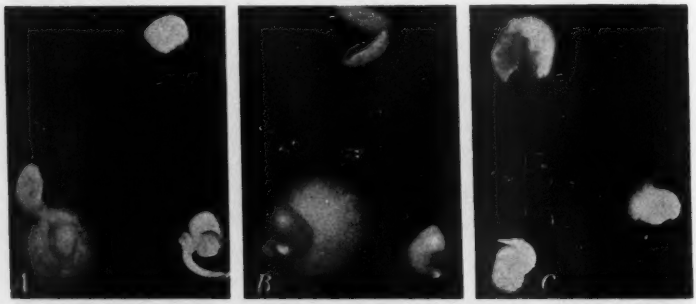


FIGURE 3.—Seeds from near-wilt-infected pea plants plated on potato-dextrose agar. The seed coats and cotyledons were separated before plating: *A*, Fungus present only in seed coat; *B*, fungus present only in cotyledon; *C*, fungus present in both seed coat and cotyledon.

were planted at Moscow, Idaho, on soil where near-wilt had never been observed. Out of 140 plants of Wisconsin Perfection, 16 became diseased with near-wilt, and out of 656 plants of Prince of Wales, 10 were diseased. Fifty-four pea crosses, mostly backcrosses from small-podded, somewhat resistant lines to near-wilt-susceptible, large-podded varieties, were made in the greenhouse on near-wilt-infested soil. Most of the plants on which the crosses were made set one or two pods before dying from near-wilt. The 54 crosses furnished about 140 seeds, which were planted the following spring in the plots at Moscow. Four of the resulting plants became affected with the disease. These cases of near-wilt were the only ones found in the plot, which covered about one-fifth of an acre.

TABLE 1.—*Results of plating seeds collected from near-wilt-diseased plants*

Variety	Seeds tested	Seeds infected
	Number	*Number*
Prince of Wales	70	2
Wisconsin Perfection	70	1
Penin	141	21
Senator	50	1

The reults of the isolation experiments leave little doubt that the diseased plants developed from infected seed produced by susceptible plants grown on near-wilt-infested soil.

DISCUSSION

Penetration of the root tip of pea plants by the near-wilt organism is quite similar to penetration of root tips of China-aster plants by *Fusarium conglutinans* Wr. var. *callistephi* Beach as described by Ullstrup (*9*), and the penetration of cabbage plants by the cabbage yellows organism (*Fusarium conglutinans* Wr.) reported by Smith and Walker (*4*). The near-wilt organism differs, however, from these strictly vascular parasites in that it may also invade the cortex extensively. In this respect it is similar to the watermelon wilt organism (*Fusarium niveum* Erw. F. Sm.). No explanation is offered of why the fungus invades the cotyledonary region so readily, unless it could be that the organism in contact with a germinating seed grows over its surface and immediately attacks the cotyledonary region when the young seedling breaks through the seed coat. What conditions are necessary for the organism to cause a rot are not definitely known, but it does seem that an abundance of mycelium must be present on the surface of the seedling to initiate it. Results quite similar to those reported by Appel (*1*) were obtained when the seed was sprayed with a suspension of macroconidia of the near-wilt fungus.

The ability of the organism to travel the complete length of the stem of susceptible pea varieties is an outstanding difference between this disease and the wilt disease described by Linford (*2*), who never found the wilt fungus (*Fusarium orthoceras* App. and Wr. var. *pisi* Linford) within two nodes of the lowest blossoms of susceptible varieties. It has been shown in this investigation that penetration takes place equally well in Wisconsin Perfection and Rogers K, but that the fungus does not advance nearly so far up the stem in the latter as in the more susceptible varieties. No morphological differences were observed which would account for this difference. Perhaps some substance is present in Rogers K which slows down the development of the fungus, and it therefore could progress only so far before the plant matured.

It seems logical to assume that certain varieties are more liable than others to produce infected seed when grown on infested soil. Early-maturing types, such as Alaska, often mature before the organism can reach the pods, whereas in later maturing ones like Perfection and Penin the organism has a much longer time to travel approximately the same distance to reach the seed. It is doubtful if the organism could reach the seed of very tall varieties of peas by way of the vascular system except under very favorable conditions. Although only a small percentage of seeds carry the fungus, there is ample opportunity for it to become widely disseminated. Thus, it is possible that the near-wilt disease may at some time become a more serious menace to the pea industry than it has up to the present.

SUMMARY

The near-wilt fungus (*Fusarium oxysporum* Schlect. f. 8 Snyder) makes its entry into the pea plant at almost any point along the root and epicotyl of young seedlings, the root tip and cotyledonary node being the most common points of entry. No difference in penetration of Wisconsin Perfection, a very susceptible variety, and Rogers K,

a somewhat resistant variety, was observed. Under certain conditions the fungus was able to produce a definite seedling root rot.

The progress of the fungus through the root and stem was largely confined to the xylem vessels. The organism was able to travel the complete length of stems of many susceptible varieties. In the case of Rogers K and some hybrids of which Rogers K was one of the parents, the fungus failed to advance nearly so far up the stem as in the susceptible varieties.

The organism advances into the seeds of diseased plants through the vascular system. The fungus was found both in the seed coat and cotyledon. Dwarf, late-maturing, susceptible varieties are much more likely to produce infected seed when grown in near-wilt-infested soil than are early-maturing varieties. Seed infected by the near-wilt organism will produce diseased plants.

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PRELIMINARY STUDIES OF THE NUTRITIVE REQUIREMENTS OF THE EUROPEAN CORN BORER[1]

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INTRODUCTION

Much research conducted on the European corn borer (*Pyrausta nubilalis* (Hbn.)) in relation to potential host plants, number of generations, breeding of imported parasites, and especially borer-resistant strains of corn has emphasized a growing need for accurate knowledge of the nutritive requirements of the insect.

After a certain strain or variety of any plant has been proved to possess some resistance to the attack of an insect, the possibility of breeding this resistant quality into new strains of the plant that are adapted to different localities and purposes would appear far more encouraging if the plant breeder could be furnished definite knowledge of the insect's nutritive requirements. A working knowledge of the inherent characters or factors which constitute or contribute to the resistance of a particular variety of plant to attack by a certain insect would permit the immediate elimination of many varieties or strains of the plants from extensive but futile tests of resistance. Definite information concerning the physiological effect of nutrients on an insect might also aid in forecasting the response of the insect's parasites when the food plant of the insect is growing in a different environment, or the magnitude that an infestation of the insect might be expected to assume on a new or different host plant.

During the spring of 1937 tests were begun at the Toledo, Ohio, research laboratory to study the nutritional requirements and limitations of the European corn borer with special attention to its ability to feed on and assimilate the tissues of different food plants. The results of these tests are reported in the present paper.

METHODS, TEST MATERIAL, AND EQUIPMENT

Each experiment for testing the effect of nutrients on the borer was replicated four times, and the material used in each replication was infested with approximately 100 eggs. For a period of 25 days the test larvae were supplied with fresh food every fifth day. Thirty days after infestation the surviving larvae were weighed and isolated for further observations on development, survival, pupation, and emergence. Although the percentage of pupation prior to diapause among ostensibly single-generation borers has been considered negligible, the percentage of larvae in these tests that pupated without diapause varied considerably with the nature of the nutritive sub-

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[2] Grateful acknowledgment of assistance is made to W. A. Baker, in charge of corn borer research, under whose leadership these studies were initiated, and to A. M. Vance, under whose general supervision the tests were conducted.

stance supplied them. Thus, in many instances the pupation of a portion of the larval population reduced the number of larvae available for weighing, and for this reason the weights of larvae as recorded from such populations may not be entirely comparable with those representing populations in which no pupation occurred. No pupae were weighed.

Supplementary tests for enzymes present in the digestive tracts of corn borer larvae were conducted subsequent to these feeding experiments in an effort to gain some fundamental knowledge concerning the insect's digestive capacities.

FOOD MATERIALS

Food materials tested were selected to represent distinct types of physical structure, differences in stage of plant maturity and in moisture content, different chemical constituents, and different strains of corn that had proved either resistant or susceptible under field tests as reported by Patch and Bottger[3] and Marston and Dibble (*6*).[4]

Before infestation the food materials were washed and cut or broken into portions which exposed a comparatively large amount of feeding surface to the borer. The fragments of food usually were laid close together to provide an environment to satisfy the borers' thigmotropism.

Proximate composition of vegetable foods tested was assumed from analyses by Chatfield and Adams (*2*) and from detailed analyses of sugars in sweet corn at different stages of maturity by Culpepper and Magoon (*3*). The interpretations involving the chemical constituents of material are based on such assumptions.

Fresh vegetables such as green beans, green peas, lettuce, and cauliflower were purchased from markets as needed. Five successive plantings of four different types of corn and of five other kinds of crop plants provided additional materials for both field and laboratory tests.

The kinds and portions of the plants tested are shown in table 1.

TEST INSECTS

Fall-collected corn borer larvae, all from one source, were isolated in glass shell vials and kept in cold storage at a temperature of 40° F. and a relative humidity of 70 to 100 percent. Moths emerging from this stock produced the eggs used for infesting all materials included in the tests.

LABORATORY CONDITIONS

A room-size incubator kept at a constant temperature of 80° F. and a relative humidity of 70 to 100 percent provided satisfactory conditions for emerging moths, oviposition, and the rearing of larvae on the foods tested. A metal rack permitted the stacking of the rearing trays within a comparatively small space and at the same time allowed free circulation of air which was maintained by the continuous operation of a 12-inch electric fan in the room.

PRODUCTION OF EGGS

A slight modification of the mating and ovipositing cage described by Bottger and Kent (*1*) was used. Essentially this consisted of a

[3] PATCH, L. H., and BOTTGER, G. T. INVESTIGATIONS OF THE VARIETAL RESISTANCE OF FIELD CORN TO THE EUROPEAN CORN BORER IN 1936. U. S. Bur. Ent. and Plant Quarantine E-416, 11 pp. 1937. [Mimeographed.]

[4] Italic numbers in parentheses refer to Literature Cited, p. 257.

3⅜-inch cardboard ice-cream container fitted top and bottom with an 18-mesh copper screen. Its inner wall was lined with waxed paper, on which the moths readily oviposited.

From 5 to 10 female moths and a like number of males were confined in each of these cages and kept in the incubator for mating and oviposition. Once every 24 hours the sheets of waxed paper bearing the egg masses were removed from the oviposition cages. The paper was then cut into pieces so that each piece bore a single egg mass. These were placed in terra-cotta dishes, covered with a glass plate, and returned to the incubator room for incubation of the eggs. The glass cover permitted the frequent observation of embryonic development, which was essential because the eggs had to be removed just prior to hatching for ease in counting and for insurance of a complete hatch after they were placed on food material.

INFESTATION OF FOOD MATERIALS

The eggs were incubated until the head capsules of the contained larvae became sufficiently pigmented to be readily visible, at which time groups of approximately 100 eggs were counted and placed on food in rearing trays, where hatching usually occurred within a few hours. The number of eggs failing to hatch was negligible.

REARING TRAYS

Two types of rearing trays were used in the laboratory tests. The one most generally employed was that described by Mathes (*7*). It consisted of a rectangular wooden frame fitted with an 80-mesh copper-screen bottom and a glass top.

For some of the more concentrated foods which contained little fiber, a terra-cotta dish 7 inches in diameter and 2 inches deep was found practical. A satisfactory lid was provided by a round piece of 80-mesh screen of the same circumference as the inside of the dish about one-fourth inch from its top. The slightly flared sides of the dish served as a shoulder on which the rough edges of the screen cover rested.

ENZYME TESTS

The entire digestive tracts from 25 hibernating larvae were ground with glycerin in a mortar to form a tissue suspension to make each test for an enzyme.

The methods of enzyme analysis used were the microchemical methods reported by Swingle (*8*).

RESULTS

The percentages of survival and pupation and the weights of test larvae of the European corn borer reared on 33 samples of plant material are presented in table 1. Each number is the average of four replications.

These data were analyzed by the method of the analysis of variance described by Fisher (*4*). The required difference for significance between treatment means was calculated from the remainder variance and from the value of t obtained from Fisher's table of t, in which the number of degrees of freedom applicable to this problem for a probability of 0.05 was used. The significance of the correlation

coefficient was determined by computing t from the correlation coefficient and the number of pairs of observations on which the correlation was based, less 2. The probability of such a value was then ascertained from Fisher's table of t.

TABLE 1.—*Effect of certain nutrients on survival, weight, and pupation of European corn borer larvae, arranged in decreasing order of survival*

Plant	Portion of plant fed larvae for 30 days	Survival of test larvae	Average weight per larva	Larvae pupated [1]—		
				30 days after hatching	40 days after hatching	60 days after hatching
		Percent	*Milligrams*	*Percent*	*Percent*	*Percent*
Sweet corn (*Zea mays*)	Kernels	65.3	66.6	21.7	38.6	41.9
Green beans (*Phaseolus vulgaris*)	Pods and fruit	51.6	96.0	9.6	12.5	12.5
Popcorn, Japanese hull-less	Internodes	47.1	92.9	8.8	10.8	11.4
Dent corn, A × Tr	do	43.5	100.1	17.2	23.6	27.0
Sweet corn, bantam	do	41.1	120.1	13.5	15.3	16.0
Dent corn, Hy × R4	do	33.0	78.3	11.4	15.9	19.7
Lettuce (*Lactuca sativa*)	Leaves	32.0	83.2	0	1.6	1.6
Flint corn, maize Amargo	Internodes	30.1	124.4	1.9	2.9	2.9
Green peas (*Pisum sativum*)	Pods and fruit	21.2	92.6	3.5	3.5	4.7
Sorghum (*Holcus sorghum*)	Leaves and stem	18.4	102.8	7.9	14.4	14.4
	Internodes	14.6	67.4	10.0	10.0	10.0
Sweetpotato (*Ipomoea batatas*)	Roots	12.5	43.6	2.0	3.9	3.9
Sunflower (*Helianthus* sp.)	Leaves and stem	10.3	25.0	0	0	0
Smartweed (*Polygonum* sp.)	do	8.9	62.2	2.7	5.0	5.0
Cocklebur (*Xanthium* sp.)	do	8.1	71.1	3.3	10.0	10.0
Table beet (*Beta vulgaris*)	Roots	7.4	80.5	0	0	0
Dent corn, A × Tr	Leaves	6.9	73.1	13.8	13.8	13.8
Flint corn, maize Amargo	do	6.9	53.2	17.9	17.9	17.9
Sunflower	Sections of stalk	5.4	31.7	0	0	0
Giant ragweed (*Ambrosia trifida*)	Leaves and stem	5.4	46.0	0	0	0
Asparagus (*Asparagus officinalis*)	Young shoots	5.0	53.6	0	0	0
Banana (*Musa sapientum*)	Fruit	4.1	62.8	0	0	0
Cauliflower (*Brassica oleracea botrytis*)	Flower	3.9	73.2	11.1	11.1	11.1
Sweet corn, bantam	Leaves	3.9	32.0	18.8	18.8	18.8
Dent corn, Hy × R4	do	2.5	55.8	0	0	0
Popcorn, Japanese hull-less	do	2.4	28.7	[2] 42.9	[2] 42.9	[2] 42.9
Hemp (*Cannabis sativa*)	Leaves and stem	1.9	26.1	0	0	0
Potato (*Solanum tuberosum*)	Tubers	.7	51.1	[2] 33.3	[2] 33.3	[2] 33.3
	Leaves and stem	.5	17.8	0	0	0
Dent corn, Hy × R4	Tassels	0				
Dent corn, A × Tr	do	0				
Soybean (*Soja max*)	Leaves and stem	0				
Table beet	Leaves	0				
Required difference for significance at odds of 19 to 1.		13.1	34.1	32.1		

[1] Based on numbers of larvae surviving.
[2] Extremely low larval survivals resulted in populations too low for reliable comparison with percentages of pupation occurring among the other populations.

SURVIVAL

On the basis of the required difference of 13.1 percent for odds of 19 to 1, the 65.3 percent of larvae which survived on a diet of sweet corn kernels was significantly greater than the percentage which survived on any other food tested.

The survival of 51.6 percent of larvae nourished on green beans was next highest to the survival on sweet corn kernels and was significantly higher than any survival of 38.5 percent or less. The survivals of 47.1, 43.5, and 41.1 percent on the internodes of popcorn A × Tr dent corn, and bantam sweet corn, respectively, were significantly higher than any survival of 28 percent or less. The 43.5 percent survival of larvae nourished on the internodes of A × Tr was significantly higher than the 30.1 percent survival on the internodes of the flint strain, maize Amargo, but not significantly higher than the 33

percent survival on the dent Hy×R4 internodes. However, the survival of 47.1 percent on popcorn internodes was significantly higher than the survival on either of the more resistant strains Hy × R4 and maize Amargo.

The 32-percent survival of larvae on lettuce compared favorably with that on the internodes of the two resistant strains of corn, all of which were significantly higher than any survival falling at or below 17 percent.

Survivals of 21.2 and 18.4 percent on pods and fruit of green peas and on leaves and stem of sorghum, respectively, while significantly higher than any falling at or below 5.3 percent, represented the lower brackets of survivals indicative of satisfactory nutrition.

Corn leaves (except from maize Amargo and from the strain A × Tr), corn tassels, beet leaves, hemp plants, potato plants, potato tubers, and soybean plants were foods on which either no survival resulted or an extremely low one (significantly lower than survival of 17 percent or more).

WEIGHTS

It is apparent that larvae reared on most of the corn internodes, green beans, and green peas attained greater weights than did those reared on other foods. The average weights of 124.4 and 120.1 mg. per larva nourished on maize Amargo and bantam sweet corn internodes, respectively, were significantly greater than the average weight per larva of 78.3 mg. for those reared on the internodes of Hy × R4.

The average weights of larvae reared on each of the five kinds of corn leaves (except those of A × Tr), sunflower plants, and hemp and potato plants were significantly less than the average weights of larvae reared on each of the five kinds of corn internodes (except those of Hy × R4), sorghum plants (leaves and stem), green beans, and green peas.

Statistically there is a high correlation between percentage of survival and average weight per larva 30 days after hatching. The correlation coefficient of 0.635 was calculated from 29 pairs of observed values.

PUPATION

Statistical analyses of the percentages of pupation are of little value because of the widely different populations of surviving larvae on which the analyses were based. However, excepting the instances where extremely low larval survivals resulted in populations too small for reliable comparison, relatively high percentages of pupation are associated with relatively high percentages of survival and weights of mature larvae.

The 38.6 percent of larvae reared on sweet corn kernels which had pupated 40 days after hatching exceeded the pupation for all comparable groups, again placing this nutrient in a unique position, when compared with other nutrients, in relation to pupation.

ENZYMES

Table 2 summarizes the results of tests for enzymes in the digestive tracts of corn borer larvae. The positive reaction of tissue suspensions with sucrose, olive oil, fibrin, and peptone suggests the borer's ability to digest sucrose, fat, and proteins. The pepsin reaction may have been affected by the presence of excess acid in the solution. Pepsin

is active only in a strongly acid solution, and this necessitates a more careful adjustment of the pH value than available equipment would permit. However, the reaction indicating the presence of erepsin was so pronounced that its presence could scarcely be doubted. In the presence of erepsin, peptones are broken down into amino acids, indicating the borer's ability to digest these derivatives of protein matter. The borer's inability to digest starch, maltose, and lactose is indicated by the negative reaction of the tests for amylase, maltase, and lactase.

TABLE 2.—*Summary of results of tests for enzymes in the tissues from walls of the entire digestive tracts of European corn borer larvae*

Enzyme sought	Substrate used	Tests made	Enzymatic reaction
		Number	
Amylase	Cornstarch	4	Negative
Maltase	Maltose	1	Do.
Invertase	Sucrose	4	Positive.
Lactase	Lactose	1	Negative.
Lipase	Olive oil	2	Positive.
Pepsin	Fibrin	4	Do.
Erepsin	Peptone	4	Do.

DISCUSSION

VEGETABLES

The data indicate that the nutritive elements available in immature sweet corn kernels were better adapted to the borer's requirements, when measured by survival and pupation, than those in any other food tested. Green beans, lettuce, and green peas were fairly satisfactory nutrients from the standpoint of survival and larval weight, but they did not affect the metabolic acceleration of the test insects as expressed in percentage of pupation nearly so much as did the corn kernels.

The low survivals of larvae on the other vegetables, accompanied by low weights in some instances and either low or no pupation in all instances, may have been due to mechanical resistance to attack offered by the physical structure of the plants, such as hard epidermis, or to retention of too much or too little moisture under laboratory conditions, deficiency in certain chemical constituents, or the occurrence of these constituents in a form not readily digested by the borer.

Considering the borer's favorable physiological reaction to nutrients which are relatively high in glucose, such as immature sweet corn kernels (*3*), compared with its reaction when fed foods such as beet leaves, in which practically all sugar present is sucrose, and potato tubers, which are high in starch, it seems probable that the character of the carbohydrate constituents of food may be of real importance in determining its nutritive value to the borer. On the basis of these indications, it seems logical to assume that glucose fulfills the insect's nutritive requirements far more effectively than does either sucrose or starch. Failure to obtain a favorable growth response of larvae to predominantly starchy food materials is probably due to the inability of the insect to digest starch, as was indicated by the negative reaction of the tests for amylase, the starch-splitting enzyme.

CORN INTERNODES

Relatively high survivals of larvae resulted from infestations on the internodes of all strains of corn and were accompanied by favorable rates of development in most instances, probably because of the comparatively high percentage of digestible carbohydrate constituents in green corn internodes. The differences in survival and weight of larvae between populations nourished on internodes of different strains indicate that the nutrients present which are best adapted to the borer's requirements vary considerably in amount as between different strains. That these nutrients do vary was further substantiated by physical differences in the excrement of the test larvae feeding on internodes from different strains of corn. Excrement of larvae which were feeding on internodes of the resistant strain Hy × R4 was coarse-textured and retained the original color of the food. In contrast, the excrement from larvae feeding on internodes of the susceptible strain A × Tr was fine-textured and greatly changed in color, a condition which would indicate a much more complete digestion.

The low survivals and weights of larvae confined on corn leaves are attributed to causes of a mechanical rather than a nutritive nature, and to the fact that the leaves became very dry during the interval between feedings. Since the thick portion of the midribs and a small area of the leaf and sheath adjacent to the ligules were the only portions of the leaves which appeared to satisfy the positive thigmotropism of the borer, it seems probable that the majority of larvae were unable to find an environment conducive to their normal activities and subsequent development. Both etiolated and chlorophyllaceous leaf tissues were supplied the larvae in these tests.

The fact that no larvae survived on the tassels of the two strains of dent corn was contrary to expectations, because the tassel is one of the most favorable points of entrance, and its availability has been commonly associated with high borer survivals in the field. While newly hatched larvae appeared to feed normally on the tassel spikelets, any feeding observed on tassels under test after the larvae were 5 days old was confined to the main stem, and as soon as that source of nutrition was eliminated, either by larval feeding or by desiccation, the larvae died without any apparent attempt to feed on the spikelets. Corn tassels dried rapidly after they were placed in rearing cages, and this may have caused a large portion of the larval mortality.

The close similarity between the carbohydrate constituents of leaves, tassels, and stalks of green corn plants, as shown by the analysis of Latshaw and Miller (*5*), makes it unreasonable to assume that carbohydrate nutritive values were responsible for the marked physiological differences in test larvae nourished on them.

MISCELLANEOUS CROP PLANTS AND WEEDS

The total mortality of larvae confined to soybean plants appears to have been due largely to the mechanical barrier afforded by the profuse pubescence peculiar to all aerial portions of these plants. Many dead first-instar larvae were observed which had become lodged between the bristly hairs composing the pubescence.

Thick layers of coarse fibrovascular bundles were observed to surround the pith of sunflower, hemp, and giant ragweed plants and are believed to have contributed to the resistance of such plants, because relatively low survivals and weights of larvae were associated with

plants in which the fibrovascular character was prominent. High water content of the sunflower stems apparently prevented the normal tunneling and feeding of the few borers which were successful in gaining entrance through the bristly pubescence and hard epidermis.

Neither the physical structure nor the nutritive qualities of cocklebur, smartweed, giant ragweed, or potato plants could be considered favorable to the establishment and development of the borer, on the basis of its physiological reactions to any of them. The physical changes which occurred in these plants under laboratory conditions doubtless contributed to their unsatisfactory nutritive qualities.

With the sorghums, weights of larvae reared on internodes were significantly lower than of those reared on leaves and stems of much younger plants, thus indicating that the changes in these plants, probably both physical and chemical, which occur with maturity affect the borer adversely.

ENZYMES

The absence of amylase (starch-splitting enzyme) in the borer's digestive tract as indicated by these tests is offered as a possible explanation of low survivals and weights of larvae which resulted when they were confined to starchy food materials such as mature potato tubers. The presence of protein-splitting enzymes such as pepsin and erepsin is in keeping with favorable survivals and growth rates of larvae reared on foods such as green beans, green peas, and green corn kernels, all known to be relatively rich sources of protein. A pronounced positive reaction for sucrase leaves little doubt of the presence of this enzyme in the walls of the borer's digestive tract. Obviously the more favorable growth rate of larvae on foods relatively rich in glucose than of larvae reared on foods rich in sucrose, but containing little glucose, cannot, on the basis of these tests, be attributed to the nonexistence of sugar-splitting enzymes.

SUMMARY AND CONCLUSIONS

During the spring of 1937 a study of the nutritional requirements of the European corn borer (*Pyrausta nubilalis* (Hbn.)) was undertaken at Toledo, Ohio. The food materials tested were selected to represent distinct types in physical structure, stage of maturity, moisture content, chemical constituents, and both resistant and susceptible strains of corn. Their proximate composition was assumed from analyses by Chatfield and Adams and Culpepper and Magoon.

Moths that emerged in the laboratory from one lot of corn borer larvae collected in the field the previous fall supplied the eggs and resulting larvae used for all the tests. A room-size incubator kept at 80° F. and a relative humidity of 70 to 100 percent was used for the emerging moths, oviposition, and the rearing of larvae on the foods.

As measured by statistically significant differences in percentage of survival and by the relatively high percentage of pupation of test larvae, the sweet corn kernels satisfied the borer's nutritive requirements better than any other material.

Comparatively high percentages of larvae survived on green beans, lettuce, and green peas, and the comparatively high weights of larvae nourished on these foods indicated that they provided satisfactory nutrition.

Internodes of corn in general provided satisfactory nutrients for the borer. The nutritive qualities of corn internodes, as measured

by differences in survivals and weights of corn borer larvae nourished on them, varied considerably as between the strains tested.

Extremely low survivals resulted from infestations induced on potato plants and mature tubers, hemp, and sunflower plants. Soybean plants, corn tassels, and beet leaves were the only green-plant food materials tested in the laboratory on which no larvae survived, although in the case of corn tassels mortality may have been due to the poor condition of the food.

Food materials rich in glucose fulfilled the borer's nutritive requirements far better than did those high in either sucrose or starch.

Physical characters of plants which were associated with low survivals and weights of larvae were profuse pubescence, thick epidermis, and the greater number and coarse nature of fibrovascular bundles, especially in the peripheral region of the stems.

Excessive moisture in such succulent foods as young sunflower plants and potatoes apparently interfered with the tunneling habits of the insect. Insufficient moisture to satisfy physiological requirements of the insect and physical structure of the plant tissue incompatible with the positive thigmotropism of the insect probably contributed more to larval mortality on such foods as corn leaves and tassels than any deficiencies in nutritive elements.

A positive correlation was found between survival of larvae and their average weights 30 days after hatching.

Supplementary tests for enzymes in the cellular tissue of the digestive tracts of hibernating larvae indicated the presence of both sucrose- and protein-splitting enzymes, but no enzymes capable of hydrolyzing starch were found.

Metamorphic development of the insect was directly affected by nutrition. This observation suggests a possible explanation for the variation in number of generations, within a climatic range, on the basis of difference in the nutritive value of plants available in different environments.

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PHYSIOLOGICAL STUDIES OF PLASTID PIGMENTS IN RINDS OF MATURING ORANGES[1]

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INTRODUCTION

Growers of citrus fruits have long been aware that rind color is not necessarily an index of dessert quality. Early-maturing varieties of oranges are quite edible before they have lost their green pigment. In later varieties, such as Valencia, this color change occurs long before the fruits become suitable for consumption and they may regreen during the normal harvest period. Fruits of late varieties sometimes fail to lose their green color because of shade or other local conditions and may pass into senility and finally drop from the tree without having shown the characteristic orange color. The latter aspect becomes of increasing commercial significance along with increasing age of the grove.

For many years it has been customary to remove the green rind color, under conditions indicated above, by the use of ethylene. The process whereby the fruit loses this green pigment (chlorophyll) is termed "degreening," whether it is permitted to take place on the tree or whether it is hastened by the use of ethylene. Frequently difficulties are encountered, as a result of which the fruits, though legally mature, cannot be properly degreened for the market. Although many improvements have been made in the mechanical equipment employed in degreening fruit, little is known concerning physiological changes taking place in the rind during the degreening process. The present work was inaugurated in the hope that a detailed investigation of these processes might help solve some of the present-day problems in the industry.

REVIEW OF LITERATURE

While a number of investigators have identified the carotenoid pigments occurring in citrus fruits, only a few have studied the pigment transformations during degreening of the fruit or the influence of ethylene on this process. Gardner (*4*)[3] demonstrated the presence of a chlorophyll-decomposing enzyme in the rinds of satsuma oranges. Stahl (*11*) reported a slight increase in carotenoid pigments in orange rinds during maturation. Chace (*1*) stated that "the changes produced by ethylene gas are solely those which would have been brought about by nature in a somewhat longer time." Crocker et al. (*3*) suggested that since ethylene is a product of the metabolism of plant

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[2] The writers are indebted to D. F. Fisher, of the Division of Fruit and Vegetable Crops and Diseases, for helpful suggestions in planning these investigations, to C. J. Thompson, of the same Division, for constructing and assisting in adapting the Clifford photometer, and to P. A. Clifford, of the Food and Drug Administration, U. S. Department of Agriculture, for supplying specifications for the light filters. Appreciation is expressed also to Ward B. Matthews, Doris Prophitt, and Robert Bunch, of the Division of Fruit and Vegetable Crops and Diseases, for assistance in the analytical work.
[3] Italic numbers in parentheses refer to Literature Cited, p. 267.

organs it might act as a fruit-ripening hormone. Michener (*6*) stated that the effects of ethylene on growth are to be explained not as direct effects of ethylene alone, but as effects of ethylene on a growth hormone. Lynch (*5*) formulated the theory that ethylene acts as a coenzyme in the ripening processes of fruits.

MATERIALS AND METHODS

Studies were conducted during the 1936–37 citrus season at Orlando, Fla., and continued during the 1937–38 season at the Arlington Experiment Farm, Arlington, Va. The fruit used during the second season was shipped by express from Florida.

Parson Brown [4] oranges were obtained from what is known as the Carney grove at Oklawaha, Marion County, one of the original plantings of this variety. This grove is located in the northern fruit section of the Florida citrus belt, on "high hammock" soil originally supporting a growth of hardwood trees. The rootstock was sour orange. The Parson Brown orange was introduced by J. L. Carney, former owner of this grove, the budwood having been selected from what was considered the best of five promising seedling trees in the grove of "Parson" Brown, about 30 miles south. This variety is one of Florida's early oranges, in season during October and November.

Pineapple [4] oranges were collected in the Crosby-Wartman grove at Citra, Marion County. The grove is situated in a "low hammock," and there is a limestone formation a few feet under the soil. The original growth was mostly hardwood. This grove is nearly at the northern limit of citrus production on a commercial scale. The trees were budded in place on wild sour orange trees found growing in the uncleared forest. Budwood for the trees in this grove came from the original Pineapple orange tree,[5] produced in the grove of J. B. Owens, about 5 miles from Citra. The season for pineapple oranges is December to February.

Valencia [4] oranges were obtained from a grove belonging to the Tampa Forwarding Co., 2 miles southeast of Dundee, in Polk County. The grove is located in the "ridge section" of Florida, on what is locally known as "high pine sandy soil." The trees are on rough lemon rootstock. The Valencia season extends from about March to June.

Temple [6] and tangerine [7] oranges were all collected from commercial groves near Orlando.

Ten trees in each of the groves described above were reserved for the experimental work. Samples of Parson Brown and Pineapple oranges were collected every 3 weeks, and Valencia samples were taken every 4 weeks. Only 2 of the 10 trees were reserved for the few samples taken after the commercial picking dates.

Before an intelligent investigation can be made of pigment transformations induced in citrus rinds by ethylene treatment, it is essential to know what changes take place in nature. The most obvious effect of degreening is the loss of chlorophyll. However, an accurate appraisal of changes in carotenoid content cannot be made without recourse to chemical analysis.

[4] Parson Brown, Pineapple, and Valencia are varieties of *Citrus sinensis* (L.) Osbeck.

[5] The writers are indebted to W. J. Crosby, of Citra, Fla., for these historical data.

[6] Origin unknown; supposed to be a natural hybrid with *Citrus nobilis* var. *deliciosa* (Tenore) Swingle as one of the parents.

[7] *Citrus nobilis* var. *deliciosa*.

Thirty representative fruits were selected for individual samples. The fruits were peeled with a sharp scalpel, care being taken to remove both albedo and flavedo without rupturing the juice sacs of the pulp. The peel was ground in a food chopper. The procedure followed in the extraction of the plastid pigments was essentially that recommended by Schertz (*10*). Duplicate 20-gm. samples of the ground peel were covered with a 1-percent solution of sodium carbonate (Na_2CO_3) and ground to a paste with clean quartz sand in a mortar. The sample was then transferred quantitatively to a Büchner funnel, pure acetone being used to rinse the mortar. The paste was extracted with acetone until the extracts were colorless. The combined extracts were taken up with ether, treated with a little 1-percent sodium carbonate solution, and washed repeatedly with water until the ethereal solution was free from flavones. Five milliliters of absolute methanol saturated with potassium hydroxide (KOH) was added to the extract, which was shaken and then stored in the refrigerator overnight. The next day the chlorophyll, which had been saponified by the treatment described, was washed out with water. Schertz's precautions for complete separation of the chlorophyll from the carotenoid pigments were observed by rewashing each fraction until the particular solvent was colorless. The solution was made up to volume at 20° C., and its chlorophyll content was determined colorimetrically.

The ethereal solution of carotenoid pigments was dehydrated with anhydrous sodium sulfate and evaporated to dryness at a temperature below 50° C. At this place in the procedure Peterson's method (*9*) for the separation of carotene from xanthophyll was substituted for that of Schertz. The petroleum-ether fraction (cryptoxanthin, carotene)[8] was made up to 100 ml., and the methanol fraction was made up to 200 ml. The pigments were determined colorimetrically.

Colorimetric determinations were made with a Clifford (*2*) photometer.[9] The instrument was adapted for this purpose by employing a red light filter for chlorophyll and a blue filter for the carotenoids. The former consisted of dark-shade pyrometer red, Corning No. 241, transmitting from 630 mμ up. The blue filter consisted of a combination of Corning violet No. 511 and Noviol shade A No. 038, transmitting at approximately 458 mμ. The photometer wedge was standardized with solutions of crystals of chlorophyll, of beta-carotene, and of xanthophyll.[10] The same zero point could be used for each of the three solvents by compensating with clear microscope slides, thus eliminating the necessity for changing the position of the wedge in determining the three pigments. Successive readings on the same solution could be replicated within 1 or 2 mm. on the scale with a 2-inch cell. Accurate readings on pigments could be made with chlorophyll solutions as dilute as 1 part per million and with the carotenoids as dilute as 1 part in 20 million. All solutions were made up to volume at 20° C., and the readings were made as soon there-

[8] In the determination of plastid pigments in green leaves it is customary to designate the petroleum-ether fraction of carotenoids as carotene and the methanol fraction as xanthophyll. Recently Zechmeister and Tuzson (*12*) have reported that cryptoxanthin is much more abundant than carotene in the fruits of the Seville orange (*Citrus aurantium* L.). In view of this report, these two carotenoid fractions will be referred to in this paper as the petroleum-ether fraction and the methanol fraction, even though the color standards employed were beta-carotene and leaf xanthophyll, respectively.

[9] Munsey (*8*) has employed the Clifford photometer for carotene determinations and suggests its use for chlorophyll and xanthophyll readings.

[10] Especially prepared for standardization by F. M. Schertz.

after as practicable, although tests made at 20°, 25°, and 30° indicated that no significant errors would result from temperature fluctuations in this range. The photometer offers the additional advantage that when it is used there is no need to employ standard solutions for comparison after the wedge has once been standardized.

RESULTS

PROGRESSIVE CHANGES IN PLASTID PIGMENTS

The progressive changes in the plastid pigments of the rinds of Parson Brown, Pineapple, and Valencia oranges are shown in figure 1.

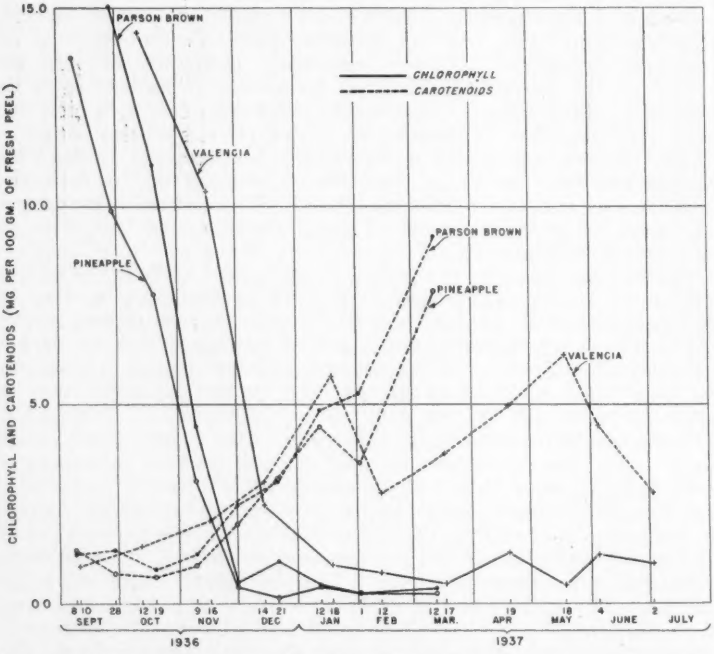

FIGURE 1.—Progressive changes in chlorophyll and carotenoids in orange rinds.

The initial samples were collected just before the fruits showed the first signs of losing their green color. From this time on the loss of chlorophyll proceeded rapidly and rather steadily. The curve for chlorophyll in Valencia oranges never reached the zero point, because of the regreening of the fruits in the spring when the trees began making new growth. Fluctuations are doubtless due to sampling errors, because all fruits on the same tree do not regreen to the same extent and these were random samples selected for uniformity of size.

From these results it appears that Parson Brown and Pineapple oranges never completely lost their chlorophyll. Whether this is the case is not definitely known. After the fruit has attained full color on the tree there is still a minute amount of pigment that is determined

as chlorophyll by the method employed. It is not known whether this is chlorophyll that is being formed as a result of the new growth and occurs in quantities too minute to be detected with the eye, or whether it is a pigment isolated along with chlorophyll but not detectable until the chlorophyll has all disappeared.

It is interesting to note that the carotenoid pigments in the rinds of all three varieties began increasing rather early in the fall and before the rind had lost its green color. The regularity of the curves is broken by the February samples. Pineapple and Valencia varieties showed a loss in carotenoids at this time, and the Parson Brown variety showed only a slight increase. However, carotenoid values reached a second peak in March for Parson Brown and Pineapple varieties and in May for Valencia. The March samples constituted the last for the early and midseason varieties, because this was long after the picking season for Parson Brown and rather late for Pineapple oranges. The Valencia oranges showed lower carotenoid content in the peel on each of the sampling dates subsequent to May. In this variety, too, the fruit had passed its most marketable stage at the time of the last sampling.

The next question arising was whether deeper colored fruits, such as tangerine and Temple oranges, show a similar increase in carotenoid content of the rinds during maturation. These particular fruits were sampled at but two intervals, (1) when mature green and (2) when full-colored (degreened on the tree). The results will be found in table 1. The green fruits of these two varieties contained a greater quantity of carotenoids than similar fruits of the *Citrus sinensis* type (Parson Brown, Pineapple, and Valencia). Nevertheless the fully colored fruits showed still higher quantities of carotenoids. The tangerine oranges doubled their carotenoid content in attaining full color while fruits of the Temple variety more than tripled theirs.

TABLE 1.—*Plastid pigment content of the rinds of mature green and full-colored tangerine and Temple oranges*

Variety and stage	Date	Total chlorophyll [1]	Total carotenoids [1]
		Milligrams	*Milligrams*
Tangerine:			
Mature green	Oct. 31, 1936	14.583	4.083
Full-colored	Dec. 9, 1936	.000	8.925
Temple:			
Mature green	Dec. 7, 1936	3.525	2.732
Full-colored	Jan. 6, 1937	.000	9.500

[1] Expressed as milligrams per 100 gm. of fresh peel.

EFFECT OF ETHYLENE ON PLASTID PIGMENTS

Mature green fruits were given ethylene treatment under conditions that simulated commercial conditions as far as possible. A coloring room was equipped to maintain a constant temperature of 27.8° C. and a relative humidity of 88 percent. The air in the room was circulated by means of a fan, and ethylene was introduced at the rate of 0.532 cubic feet per 24 hours. Carotenoid determinations were made on a sample before ethylene treatment, and on comparable samples after various periods of treatment. The results appear in table 2. In commercial practice the ethylene treatment is rarely applied for more than 2 days and under no circumstances would the application

extend to 8 days. The longer period was employed solely for the purpose of studying extreme conditions.

As will be seen from table 2, ethylene has little if any effect on the carotenoid content of orange rinds. Valencia oranges that contained 1.491 mg. of total carotenoids per 100 gm. of fresh peel at the beginning of the experiment were found to contain 1.478 mg. after 4 days in ethylene and 1.520 mg. after 8 days. In another experiment the carotenoid content of the peel of Valencia oranges was 3.090 mg. before ethylene treatment and 3.147 mg. after 14 days in ethylene. Parson Brown and Pineapple oranges showed similar results. In the last two varieties the figures for carotenoids before and after ethylene treatment show some fluctuation; but since the value increased in one case and diminished in the other, the discrepancy may have been due to sampling error.

TABLE 2.—*Effect of ethylene on carotenoid pigments in the rinds of oranges*

Variety	Period in ethylene	Total carotenoids[1]	Variety	Period in ethylene	Total carotenoids[1]
	Days	*Milligrams*		*Days*	*Milligrams*
Valencia	0	1.491	Pineapple	0	5.700
	4	1.478		4	6.220
	8	1.520		14	6.850
	0	3.090	Parson Brown	0	1.225
	14	3.147		2	.997

[1] Expressed as milligrams per 100 gm. of fresh peel.

DISTRIBUTION OF CAROTENOID FRACTIONS

The carotenoid pigments are usually present in green leaves in the ratio of approximately 2 parts of xanthophyll to 1 part of carotene, and it is believed that the carotene is manufactured first and converted into xanthophyll. It would therefore be of interest to learn in what ratio these pigments exist in orange rinds. From table 3 it will be noted that the methanol fraction predominates in the mature green fruit of each of the three varieties.

Between the time of collecting the fall and spring samples, the petroleum-ether fraction showed a much greater increase than did the methanol fraction. In the last sampling of the Parson Brown variety the petroleum-ether fraction was 18.4 times its original value. In Pineapple and Valencia varieties the petroleum-ether fraction was 27.6 and 4.3 times its original value, respectively.

TABLE 3.—*Distribution of carotenoid fractions in the rind of oranges at the beginning and end of the season*

Series	Date	Variety	Chlorophyll	Petroleum-ether fraction	Methanol fraction	Total carotenoids
			Milligrams	*Milligrams*	*Milligrams*	*Milligrams*
6200	Sept. 29, 1937	Parson Brown	4.350	0.225	1.000	1.225
6206	Feb. 1, 1938		.000	4.150	1.060	5.210
6600	Oct. —, 1937	Pineapple	5.000	.207	1.027	1.234
6606	Feb. 22, 1938		.000	5.720	2.815	8.535
6800	Nov. 11, 1937	Valencia	3.000	1.025	1.550	2.575
6804	Feb. 23, 1938		.000	4.370	1.225	5.595

It has sometimes been observed that when certain varieties of oranges attain full color the fruits on the northeast side of the tree exhibit a deeper color than those on the southwest side. Frequently, the fruit on the south or southwest side of the tree will be deeper colored on the unexposed surface. This is especially true of highly colored kinds like Pineapple, tangerine, and Temple. The peel from fruit of the above-mentioned kinds was analyzed to determine the carotenoid fraction responsible for this deepening of color in particular tree locations. The results for Pineapple oranges appear in table 4.

TABLE 4.—*Carotenoid content of peel of Pineapple oranges from different sides of the same tree and of an unevenly colored Pineapple orange*

Source of peel	Series No.	Description	Petroleum-ether fraction [1]	Methanol fraction [1]	Total carotenoids [1]
			Milligrams	*Milligrams*	*Milligrams*
Oranges from different sides of tree.	7200	Fruit from northeast side (deeper colored).	6.40	2.010	8.410
	7202	Fruit from southwest side (lighter colored).	3.42	1.400	4.820
Sectors of unevenly colored oranges.	7100	Deep-colored sector	5.05	1.975	7.025
	7102	Light-colored sector	3.07	2.240	5.310

[1] Expressed as milligrams per 100 gm. of fresh peel.

It will be readily seen that the heightened color is largely due to an increase of pigment in the petroleum-ether fraction. This fraction in the rind of the fruit from the northeast side of the tree (deeper colored) was 1.9 times that in the rind of the fruit from the southwest side. Similarly, the peel from the unexposed or deeper-colored sector of the unevenly colored fruit showed 1.6 times as much pigment in the petroleum-ether fraction as the peel from the exposed or undercolored sector. The methanol fraction showed no consistent relationship to intensity of color. In the case of tree exposure, it was higher for the deeper-colored fruit, but the reverse was true for sectors of unevenly colored fruit.

DISCUSSION

Little is known concerning the behavior of carotenoid pigments in citrus rinds, and frequent inquiries are made on the subject. Does a mature green orange contain its maximum quantity of yellow pigments, and does it attain full color merely by losing the chlorophyll? Does ethylene merely destroy the chlorophyll and reveal the yellow pigments, or does it increase the quantity of the yellow while removing the green?

In the first place it is evident that the carotenoid pigments in orange rinds are not at all static. The analyses show a slight increase in carotenoid content, even while the fruit is still green. This increase in yellow pigments continues after the chlorophyll has disappeared. In the early variety (Parson Brown) the peak of carotenoid content in the rind was not reached until March, which was long after the marketing season for this variety. The late variety (Valencia) attained its first peak of full color in January, which was before it met the legal

standards of maturity. The carotenoids in the peel of this variety dropped in February, rose in May, and dropped again in July. From the results with both early and late varieties, it is apparent that maximum yellow color is not always correlated with maximum quality.

The effect of ethylene on green orange rinds is to stimulate degreening. Carotenoids in the peel showed no significant changes as a result of ethylene treatment. Frequently the exposure of oranges to the ethylene treatment for several days in the fall appears to enhance the yellow color. A close inspection reveals that this effect is produced by deeper-colored yellow splotches distributed irregularly over the surface of the rind. Chemical analyses indicated that there was no increase in actual carotenoid content.

The above results for oranges are quite different from those previously reported for limes, lemons, and grapefruit (*7*). In these lighter-colored citrus fruits there was a decrease in carotenoids as they degreened and this decrease occurred whether the fruit was degreened by ethylene or was permitted to degreen on the tree. With all types of citrus fruit investigated, however, the effect of ethylene appeared to be merely to stimulate chlorophyll decomposition and no change was produced in the fruit that would not have occurred in nature in a somewhat longer time.

It is interesting to note that in the earliest samples of oranges the methanol fraction (xanthophyll) of the carotenoids predominated, whereas the final samples showed a predominance of the petroleum-ether fraction (cryptoxanthin, carotene). In the unevenly colored fruit, resulting from unequal exposure to sunlight, the deeper-colored fruit or the deeper-colored side also showed a predominance of the petroleum-ether fraction. Inasmuch as cryptoxanthin is the only plant carotenoid beside the carotenes that has been reported as a precursor of vitamin A, it would be interesting to determine whether the petroleum-ether fraction also shows similar variation in the edible portion of the fruit.

SUMMARY

Adaptation of the Clifford photometer for quantitative determinations of the plastid pigments is described.

Periodic analyses were made on the rinds of early (Parson Brown), midseason (Pineapple), and late (Valencia) Florida oranges from the time they were mature green until long after their marketing season. The results show that as the chlorophyll decreased the carotenoids increased and continued to increase after the chlorophyll had disappeared.

Fully colored tangerine and Temple orange rinds contained a greater quantity of carotenoid pigments than the rinds of the mature green fruit.

Ethylene treatment of mature green oranges stimulated decomposition of chlorophyll without any significant effect on the carotenoid pigments.

In the samples taken when the fruits were mature green the methanol fraction (xanthophyll) predominated in the carotenoids in the rind. Later, when the fruit had attained its highest carotenoid content, the petroleum-ether fraction (cryptoxanthin, carotene) was much higher than the methanol fraction.

Pineapple oranges selected from the northeast side of the tree in the spring owe their superiority in color over those on the southwest side to the higher petroleum-ether fraction of the carotenoids. This is true also when there is a difference in color of the two sides of the same fruit.

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PRODUCTION OF EPINASTY BY EMANATIONS FROM NORMAL AND DECAYING CITRUS FRUITS AND FROM PENICILLIUM DIGITATUM [1]

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INTRODUCTION

It is common knowledge that mature green citrus fruits will eventually lose their green color if held long enough in air at room temperature. When mature green Key limes are shipped to market they frequently arrive at destination fully colored (yellow) and with the receptacles loosened in a manner similar to that occurring when citrus fruits are held in coloring rooms. Furthermore, it has been frequently observed in the Horticultural Field Laboratory at Orlando, Fla., that when a high percentage of decay occurs in stored lots of mature green oranges the surviving fruits degreen at an accelerated rate.

The present investigation was undertaken for the purpose of determining the reason for the aforementioned physiological phenomenon.

REVIEW OF LITERATURE

A considerable number of plant tissues have been shown to evolve a gaseous substance that causes epinasty in test plants such as the potato or tomato. It was suggested by Botjes (*1*) [3] and by Huelin (*8*), in 1933, that this gaseous substance might be ethylene. During recent years several investigators have conducted chemical tests that serve to prove the correctness of this assumption. In 1933 Kidd and West (*10*) reported experiments which showed that the physiologically active substance in apple vapors was a hydrocarbon, and the work of Smith and Gane (*13*) during the same year indicated that the substance might be an olefine or some complex hydrocarbon. In 1936 Elmer (*4*), analyzing the volatile products of apples which are absorbed by fuming sulfuric acid, concluded that the hydrocarbon was ethylene rather than one of its homologues. Hansen and Hartman (*7*), in 1935, as well as Denny (*3*), in 1938, eliminated the possibility of butylene and propylene by filtering the gaseous emanations through 87-percent sulfuric acid and removed any acetylene that might be present by absorbing the gas in mercuric nitrate and liberating the pure ethylene by adding hydrochloric acid. As early as 1934, however, Gane (*5*) had rather conclusively proved that apples evolve ethylene. The method consisted of absorbing the gaseous emanations in bromine and converting the ethylene dibromide thus formed into crystalline diphenyl-ethylene-diamine. Niederl, Brenner, and Kelley (*12*) and Niederl and Brenner (*11*), working with bananas, confirmed

[1] Received for publication November 29, 1939.
[2] The writers wish to acknowledge the assistance of A. Peyton Musselwhite in conducting these experiments.
[3] Italic numbers in parentheses refer to Literature Cited, p. 277.

Gane's results by determining ethylene as silver and copper acetylide.

It is reasonably certain, therefore, that ethylene is the gas involved in the production of epinasty by the gaseous emanation from plant tissue. The list of materials reported as showing positive results includes the fruits of the apple, peach, pear, plum, banana, avocado, loquat, tomato, cantaloup, squash, and eggplant, as well as seed, flowers, shoots, and leafy stems of certain other plants.

Cousins, as reported by the Jamaica Department of Agriculture (*9, p. 7*) in 1910, stated that gaseous emanations from oranges accelerated the ripening of bananas; to the writers' knowledge, this is the only work suggesting that ethylene might be evolved by citrus fruits. Elmer (*4*) reported no inhibition in the growth of potato sprouts when exposed to the emanations of oranges, bananas, or decayed apples.

Not a great deal is known concerning the physiological properties of emanations from fungi. Gane (*6*) found that the growth of pea seedlings was inhibited by the atmosphere from baker's yeast when the culture was grown aerobically. Although this toxic substance resembled ethylene in that it could be removed by absorption in bromine water, it did not stimulate the ripening of bananas. Denny (*2*) lists the following lower organisms as not producing epinasty: Baker's yeast, growing on sugar; *Rhizopus nigricans* Ehr., in potato-dextrose agar; and the fully expanded pileus and stalk of two species of mushrooms.

MATERIALS AND METHODS

The following citrus fruits were used in these investigations: Fully colored fruits of Parson Brown, Pineapple, Valencia, and seedling oranges (*Citrus sinensis* (L.) Osbeck); tangerines (*C. nobilis* var. *deliciosa* (Tenore) Swingle); partly colored and fully colored grapefruit (*C. grandis* (L.) Osbeck); Villafranca lemons (*C. limonia* Osbeck) and green and fully colored Key limes (*C. aurantifolia* (Christm.) Swingle). All of these fruits, except the Key limes, were grown in central Florida, either in the vicinity of Orlando or within a radius of 40 miles. Key limes were produced in Key Largo and shipped to Orlando by express. In most cases the fruit was placed under experimental conditions as soon as it arrived at the laboratory. When this was impracticable, it was stored overnight either in an open shed or in the basement of a building having no outlets for illuminating gas. All experiments were conducted in these rooms in order to eliminate possible contamination with ethylene from illuminating gas, coloring rooms, or other sources.

The following pure cultures of fungi were studied: *Diplodia natalensis* Ev., *Diaporthe citri* (Fawc.) Wolf; *Penicillium digitatum* (Fr.) Sacc.; and *Alternaria citri* N. B. Pierce. These organisms are among those most frequently isolated from fruit decaying in storage.

The technique employed for testing the citrus fruits was as follows: Potassium hydroxide pellets were first placed on the bottom of a 5-gallon wide-mouthed bottle to absorb the carbon dioxide produced by the fruit during the course of the experiment. The test plant was then supported above the potash by means of a coarse wire-mesh platform. The fruits were stacked around the test plant, which consisted of a potted tomato, potato, or sunflower plant, or a potato cutting in water. The jar was closed by means of a metal top and a

rubber gasket. The numbers of fruits used were as follows: 100 limes, 24 oranges or lemons, and 6 to 12 grapefruits. When decaying fruit was used the number was reduced to 5 or 10.

Two types of decaying fruit were used: (1) Fruit inoculated with the green mold organism (*Penicillium digitatum*) and (2) fruit showing stem-end decay caused by *Diplodia natalensis* and *Diaporthe citri*. Oranges were inoculated with green mold by washing the fruit with mercuric chloride and implanting the spores in the sides of the fruit by means of a sharp scalpel. Attempts to inoculate fruit with stem-end decay organisms were abandoned because of occasional contaminations with *Penicillium* after the skin was once ruptured. Instead, sound fruit was held in closed containers until definite and unmistakable stem-end rot lesions appeared, which were the result of natural infection. Sound fruit was selected from the same lot for the control, thus insuring identical treatments for both series. Cultures made of the decayed fruit at the close of the experiment showed approximately 50 percent of *Diplodia natalensis* and 50 percent of *Diaporthe citri*.

The fungi tested were grown on potato-dextrose agar in 1-liter Erlenmeyer flasks. The general scheme for the experiment was similar to that followed for the fruit. The metal top of the jar was removed and replaced by the open, inverted culture flask. The flask was fitted within the neck of the jar with two large rubber bands and weighted down with a brick.

RESULTS

Epinasty of potato leaves was produced by all of the citrus fruits tested, i. e., fully colored Parson Brown, Pineapple, Valencia, seedling, and tangerine oranges; partially and fully colored grapefruit; Villafranca lemons; and green and fully colored Key limes (fig. 1, *A* and *B*). During the winter when the temperature was between 15° and 20° C., approximately 12 to 24 hours was required to produce definite symptoms on the test plants. During the spring months when the temperature was around 25°, epinasty appeared within 4 or 6 hours. Similar results were obtained when potato cuttings, tomato plants, or sunflower plants were used for tests.

In several experiments potato plants were exposed separately to emanations from the peel, pulp, seeds, and juice of grapefruit. Only those plants exposed to the pulp showed epinasty. Typical oil injury was produced by the peel. Identical results were obtained with the peel, pulp, juice, and seeds of oranges. The results are shown in figure 1, *C*.

When grapefruits or oranges were inoculated with *Penicillium digitatum* [4] and tested for ethylene emanations it was found that decaying fruit produced epinasty more quickly than did the normal or sound fruit. The same was true when oranges showing stem-end rot lesions were used. Typical results with green mold decay are shown in figure 2. In figure 2, *A*, the photograph was made after 3 hours. At that time the plant in jar *c*, which was surrounded by decaying

[4] In an intraoffice communication, E. M. Harvey, of this Division, reported that he noted epinastic responses of potato plants when these were placed in the rooms of a commercial lemon-storage building, but because of the abundance of the decayed fruit present he suspected that ethylene might be evolved by the rot-producing fungi rather than by the fruit itself.

fruit, was the first to show epinasty. After 5 hours the plant with the sound oranges (fig. 2, *B*, jar *b*) showed epinasty also. The results with stem-end rot are shown in figure 3. In figure 3, *A*, the photograph was made after 4½ hours. Again, the plant in jar *c*, with the decaying fruit, showed epinasty, while the plant with the sound fruit

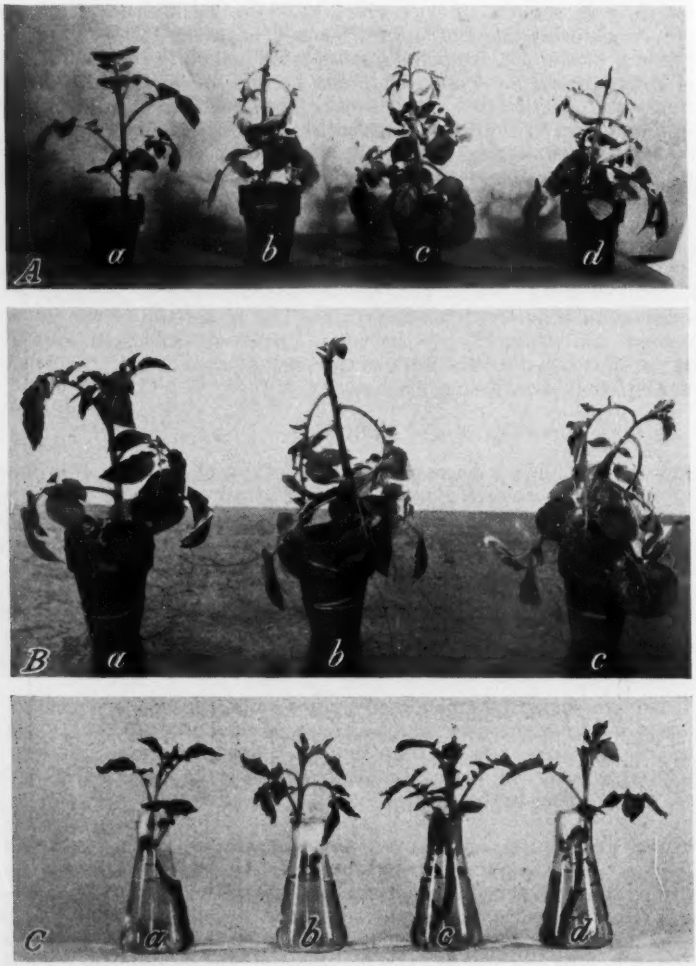

FIGURE 1.—Epinasty of potato leaves caused by emanations from oranges and limes and from various grapefruit tissues. *A: a*, Control; *b*, exposed to Parson Brown oranges; *c*, exposed to Pineapple oranges; *d*, exposed to Valencia oranges. *B: a*, Control; *b*, exposed to mature green Key limes; *c*, exposed to fully colored Key limes. *C: a*, Control; *b*, exposed to pulp of one grapefruit; *c*, exposed to seeds of one grapefruit; *d*, exposed to juice of one grapefruit.

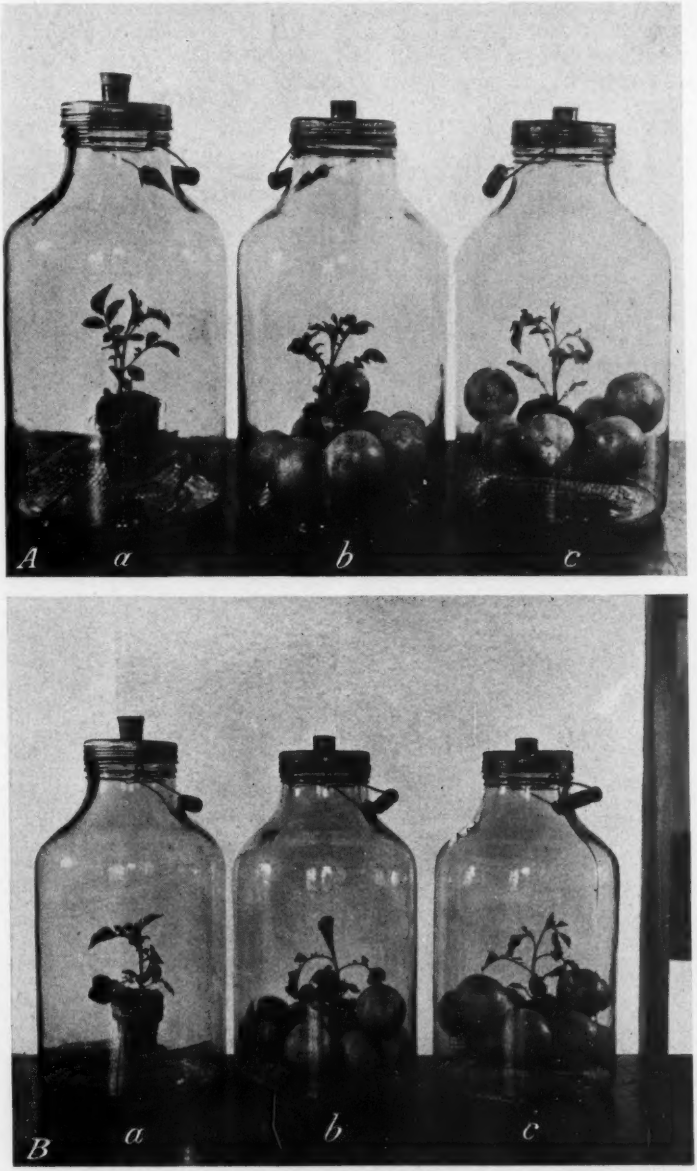

FIGURE 2.—Epinasty of potato leaves caused by sound and decaying Valencia oranges. *A*: *a*, Control; *b*, exposed to sound oranges; *c*, exposed to oranges inoculated with *Penicillium digitatum*. Photographed after 3 hours. *B*: Same as *A*, but photographed after 5 hours.

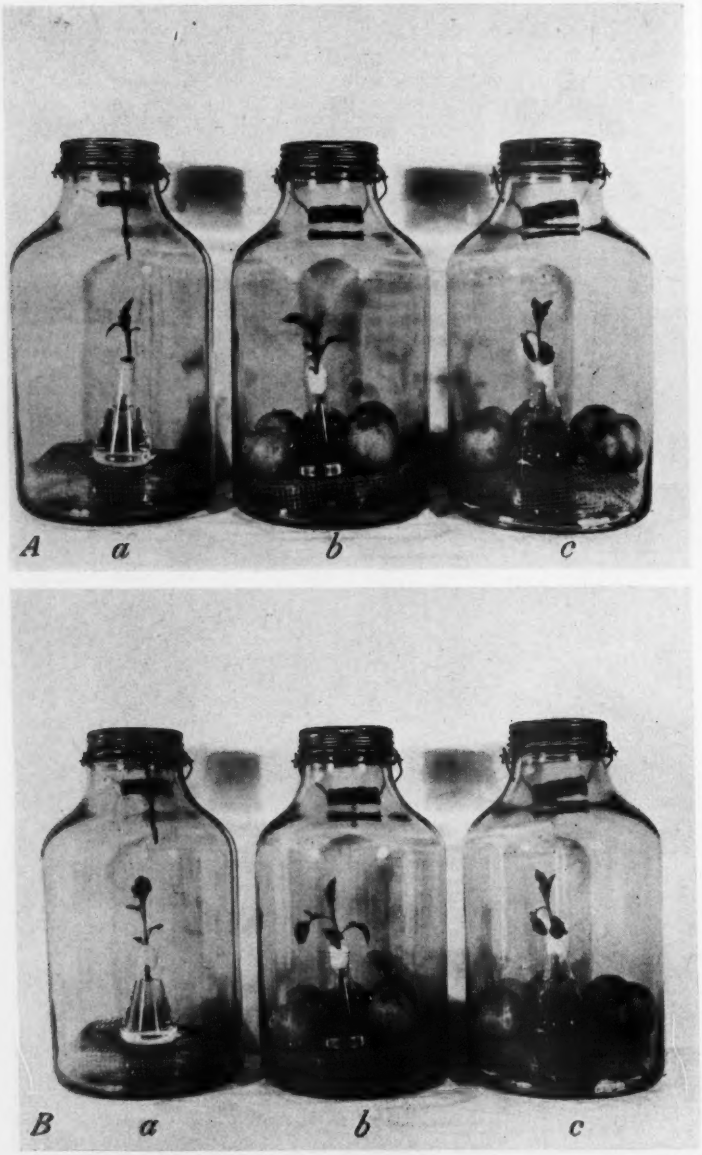

FIGURE 3.—Epinasty of potato leaves caused by emanations from sound and decaying Valencia oranges. *A: a*, Control; *b*, exposed to sound oranges; *c*, exposed to oranges showing stem-end decay. Photographed after 4½ hours. *B:* Same as *A*, but photographed after 16 hours.

(jar *b*) still appeared normal. The photograph in figure 3, *B*, was made after 16 hours. At this time symptoms of epinasty also appeared in the plant exposed to the sound fruit (jar *b*), although the effect was not so pronounced as with the decaying fruit (jar *c*). In figure 4, *A*, are shown the effects of the emanations from sound and decayed oranges on sunflower plants. After 20 hours' exposure, plant *c* (ex-

FIGURE 4.—Epinasty caused by emanations from sound and decaying Valencia oranges and from pure cultures of *Penicillium digitatum*. *A* and *B*, Sunflower plants. *A*, Effect of Valencia oranges: *a*, Control; *b*, exposed to sound oranges; *c*, exposed to oranges showing stem-end decay. *B*, Effect of *Penicillium digitatum*: *a*, Control; *b*, exposed to *P. digitatum*. *C*, Tomato plants: *a*, Control; *b*, exposed to *P. digitatum*.

posed to oranges showing stem-end rot) showed more pronounced epinasty than plant *b* (with sound oranges). Plant *a* is the control.

Evidence was obtained showing that pure cultures of *Penicillium digitatum* are capable of producing epinasty in potato, tomato, and sunflower plants. In figure 4, *B*, is shown the effect of emanations from this fungus on sunflower plants. Plant *a* is the control, and plant *b* is the treated plant. In figure 4, *C*, will be found similar results with tomato plants. Similar results were obtained repeatedly with all test plants used.

Results with pure cultures of *Diplodia natalensis*, *Diaporthe citri*, and *Alternaria citri* were either negative or inconclusive.

DISCUSSION

In view of the large number of fruits that have been shown to evolve ethylene, it is not surprising that citrus fruits may now be added to the list. From observation of the degreening of oranges, lemons, and grapefruit with ethylene, the writers have long believed that ethylene might be a natural byproduct of metabolism in citrus fruits. Earlier investigators had suggested that ethylene degreening of citrus fruits merely accomplished what was brought about by nature in a somewhat longer time. The results obtained in the present investigation tend to verify this statement.

The question of the effect of essential oils naturally arises because of the large quantities present in this type of fruit. Tests made with cold-pressed orange oil indicated that these substances were not responsible for the epinastic response of the test plants. Similar results have been reported by Denny (*3*) and Elmer (*4*).

The apparent increased evolution of ethylene by decaying fruit is a little difficult to explain. It is generally conceded that maximum evolution of ethylene by apple fruits occurs during the "climacteric." This stage in the life process of the apple is coincident with the maximum rate of production of carbon dioxide and corresponds to the period when the apple is fully ripe. It is also known that the rate of carbon dioxide production rises again when decay or break-down begins. It seems reasonable to suppose that ethylene production should increase again with the second peak in carbon dioxide production. A practical application is at once suggested. The presence of decaying oranges in a storage room may be instrumental in hastening senescence in other fruits in the same container or even in the same room.

The evolution of ethylene by fungi presents a relatively new field of investigation. Although *Penicillium digitatum* is the only organism of those studied that gave positive results, it is very probable that others may be capable of evolving ethylene. *Penicillium* is a fast-growing fungus and produces spores very readily. It is possible that during the formation of fruiting bodies a fungus, like an apple, may attain a climacteric.

A practical question is suggested here as in the case of the decayed fruit in storage. The storage life of surrounding fruits might be affected by the ethylene evolved by fungi. In view of the fact that in these experiments a relatively small quantity of the fungus produced the same effect as 24 oranges, the significance of decaying fruit in stored lots may be even greater than has been previously suspected.

SUMMARY

The production of epinasty in test plants, such as the potato or tomato, is generally conceded to indicate the presence of ethylene in the emanations of plant tissues.

Citrus fruits were tested for ethylene evolution, by using this biological method, and positive results were obtained with oranges, tangerines, limes, lemons, and grapefruit.

Oranges and grapefruit inoculated with *Penicillium digitatum* and oranges showing stem-end decay produced epinasty in the test plants sooner than did normal fruit under the same conditions.

Positive results were obtained also when pure cultures of *Penicillium digitatum* were tested for evolution of ethylene.

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A NEW VIRUS DISEASE OF SNAP BEANS[1]

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INTRODUCTION

In June 1938, while inspecting a field planting of varieties and hybrid progenies of snap beans (*Phaseolus vulgaris* L.), the writer discovered a very destructive and, apparently, undescribed disease (*4*).[3] During the growing season of 1939 the disease appeared again and was even more destructive than in 1938. This paper presents some preliminary results of a study of the symptomatology, etiology, transmission, and host range of the disease. Since the disease has thus far not been given a common name, the writer proposes the designation "black root."

REVIEW OF LITERATURE

Several virus diseases of snap beans have been described and their etiology listed as *Phaseolus* virus 1, 2, and 3 (*1, 3, 5, 6*); yet other viruses are known which, although they are classified in other taxonomic categories, produce disease on snap beans. It is this last-mentioned group of viruses that produce symptoms on snap beans most nearly approaching those of black root.

Smith (*6*), *p. 333*, states that *Datura* virus 1 causes a systemic infection in snap beans accompanied or followed by a vascular necrosis in the stem, and the entire plant is killed. He makes no mention of external symptoms or of vascular necrosis of the roots, pods, and leaves.

Nicotiana virus 12 is reported (*6, 8*) to cause necrotic spots on the leaves and necrosis of the veins on snap beans. In addition, systemic infections occur which are characterized by sunken lesions on the stems and vascular necrosis of the stem and roots. No mention is made of symptoms on the pods.

Whipple and Walker found that *Cucumis* virus 408 (*7*) produces disease symptoms on 21 species in 5 families, including 4 varieties of *Pisum sativum* and 39 varieties of *Phaseolus vulgaris*. Among the latter are varieties resistant to *Phaseolus* virus 1. Mild streaking of the stem and mottling of the leaves of young plants were produced on peas; while distinct mottling, leaf distortion, and stunting occurred on bean and, with one substrain, a severe streak. No mention is made of vascular necrosis of the roots, pods, and leaves.[4]

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[2] The writer wishes to acknowledge the suggestions given by Drs. B. B. Higgins and J. L. Weimer. He also wishes to thank M. M. Murphy, Jr., for making the photographs.
[3] Italic numbers in parentheses refer to Literature Cited, p. 288.
[4] Through correspondence with Whipple, the writer has learned that although *Cucumis* virus 408 produces some necrosis of the root and lower hypocotyl on several hosts, necrosis is not regarded as a descriptive symptom.

DESCRIPTION OF THE DISEASE

In the field, the first noticeable symptom of the disease is an incipient wilting of the leaflets, usually near the apex of the plant, during blossoming or early podding time. This symptom is too easily overlooked, however, to be of much practical importance. The first symptoms of diagnostic value appear soon after the incipient wilting and consist of a yellowing of the lower leaves followed or often accompanied by a permanent wilting of a part or all of the plant (fig. 1).

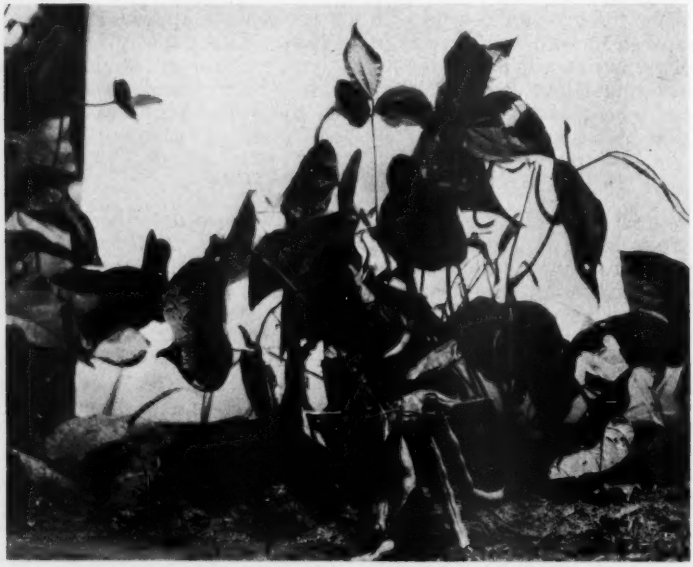

FIGURE 1.—Photograph of a diseased bean plant in the field showing early phase of wilting. Note the abnormally dark-colored sutures of the pods in the foreground.

Severe chlorosis may precede or accompany wilting, apparently dependent on or coincident with the degree of vascular involvement.

Closer inspection shows a brownish-red to purplish-black streak or streaks running lengthwise of the stem above the cotyledonary node (fig. 2, *B*, *a*).

When the epidermis and outer cortex are peeled back, or the stem is cut in cross section (fig. 2, *B*, *b*, and *C*), the streaks can be seen as an intense, dark discoloration of the area between the inner cortex and outer xylem. Strictly speaking, the inner phloem and outer xylem are most intensely discolored during the early phases of the disease, but later the discolorations may extend deeper into the xylem and even to the pith by way of the xylem rays. The external appearance of the streaks is due to the masking effect of the chlorenchyma of the cortex rather than to a surface lesion.

Well-defined external streaks are also evident on the lower hypocotyl, and, in cross section, the vascular elements are intensely discolored in this area (fig. 2, *C*). The writer has never observed any

external indication of a water-soaked condition along the streaks. Sometimes the streaks are confined to one side of the stem and the organs on the affected side die. Cross sections of such stems show the vascular discoloration to be confined to the vascular elements in one side of the stem (fig. 2, *B*, *b*). Often the discoloration may cross over to the opposite side of the stem at a node, and the plant parts free from discoloration may continue to live, at least for a time. Under such conditions of limited vascular involvement, certain plants may survive long enough to mature viable seed, a point which will be discussed later. Pole varieties often exhibit considerable discolora-

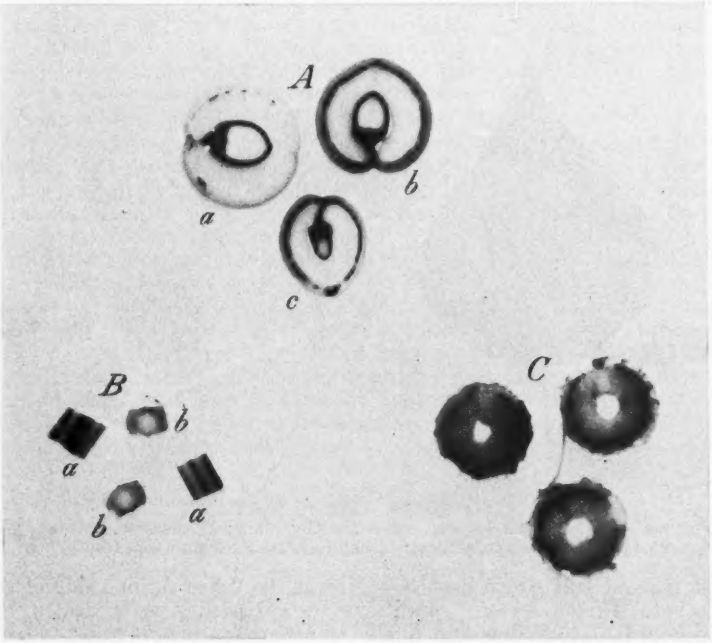

FIGURE 2.—Sections, unstained, of disease plant parts: *A*, Cross sections of diseased pods in the "snap bean" stage; *a*, Limited amount of vascular discoloration; *b*, and *c*, severe vascular involvement and mode of entrance into the seed. *B*, Plane and cross sections of diseased stems; *a*, Plane, surface view of streaks along the stem; *b*, cross sections showing fairly limited vascular involvement. *C*, Cross sections of diseased (lower) hypocotyls, showing intense vascular discoloration, inner bark, and outer xylem, as well as varying degrees of vascular flecking.

tion of the vascular elements of the median axis of the plant, but the disease is apparently confined mostly to this portion. This condition results in rapid killing of the terminal "runner," while other parts of the plant are only slightly affected, even over a considerable period of time.

Under certain other conditions of generalized infection, entire plants may wilt rapidly, apparently out of proportion to the amount of vascular discoloration usually associated with the death of plants

or of plant parts. This observation naturally raises the question as to how closely infection and discoloration may be correlated.

The diseased parts of the plant, except for the leaves and young pods, always remain firm after death, but are in a rather desiccated condition.

On the older leaves, aside from early symptoms of incipient wilting, there usually appears more or less severe chlorosis (fig. 3, *C*). The color becomes pale at first, but rapidly fades to yellow, with some green remaining in and bordering the veins. These symptoms are accompanied by a definite flagging of young leaflets, followed by a

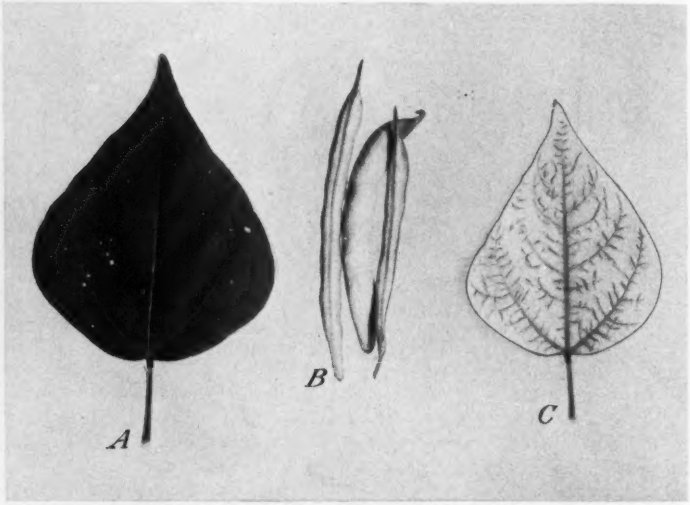

FIGURE 3.—*A*, Normal leaflet from a healthy plant; *B*, diseased pods showing the slightly darkened sutures; *C*, chlorotic leaflet from a diseased plant.

permanent wilt which usually begins at the tip or on one side of the leaflet. Under certain conditions of apparently slow death, the leaflets fade to yellow and finally to a parched brown color and fall from the plant. Under other conditions of rapid death, the leaflets wilt and dry out and curl rapidly without undergoing chlorosis. Sometimes the pulvini appear translucent but never water-soaked. The petioles do not flag but characteristically remain rigidly upright. Vascular discoloration is evident in the petioles and under certain conditions extends visibly into the veins and veinlets of the leaflets.

A careful inspection of leaflets from diseased plants, particularly when the light is favorable, shows a distinct mosaic pattern. The symptoms are not so pronounced as in the case of true bean mosaic. There is no puckering, curling, or other distortion of the lamina; only a well-defined mosaic pattern of pale and dark-green areas, more or less in a checkerboard pattern. On standing in water, or when wet by rain or heavy dew, the light areas become water-soaked in appearance.

The streaks also enter the peduncles and pods, but on the pods external symptoms appear only along one or both sutures; never

elsewhere. Here, the chlorenchyma again masks the color, causing it to appear as a slight anthocyanescence (fig. 3, *B*). When the chlorenchyma is removed, the vascular elements of the sutures and the lateral pod walls are intensely and more or less generally discolored, depending on the degree of infection. Cross sections of the diseased pods show a variable distribution of vascular discoloration (fig. 2, *A*, *a*, *b*, *c*). When young pods become infected they shrivel considerably, finally become extremely hard and brittle, but do not rot. In cross section they appear intensely discolored throughout. Somewhat older pods, in the snap-bean stage, generally show a more open distribution of vascular discoloration, and when severely infected the "pulp" sometimes presents an inky appearance as a result of its translucence and the reflection of the vascular discoloration. Young seed in severely infected pods are blasted. They often present a slimy appearance but do not rot.

Vascular discolorations can also be traced into the seed by way of the funiculus (fig. 2 *A*, *b*, *c*). If the plant and pods are not too severely infected, viable seed are often matured. White seed coats show external yellowish discolorations while self-colored seed coats, particularly black ones, become severely flecked with rust or deep buff-colored areas, i. e., color breaks.

Externally, diseased roots show no other evidence of infection than a generalized darker color, although in severe cases the taproot may appear almost black. Here, as on the above-ground parts of the plant, not all members of the root system are affected. The taproot, however, never escapes. In cross sections of diseased roots vascular discoloration is even more striking than in the above-ground parts, owing, perhaps, to the fact that there are relatively more vascular elements in the somewhat older roots and xylem discoloration is more extensive. Roots that have apparently been dead for some time do not rot.

PATHOLOGICAL ANATOMY

As stated above, the first visible evidence of vascular involvement appears as a brown to almost black discoloration of the tissues lying between the inner cortex and the outer xylem. The cambial area is an exception, at least for a considerable period after the other elements are quite discolored. In the upper stem, the discoloration does not usually extend very deep into the xylem, while in the lower hypocotyl and taproot extensive flecking may finally extend deep into the wood and even follow the xylem parenchyma almost into the center of the axis. Thin cross and longitudinal sections of diseased tissues show no evidence of a morphological break-down of any tissues, but gummy substances are present, as well as tyloses, particularly in the xylem vessels. The discoloration appears to be located principally in the walls of the affected vascular elements. Where gum and tyloses are present they are usually discolored.

From the evidence in hand, it appears that the degree and rapidity of wilting of diseased plants, while contingent on the severity of infection, is primarily correlated with the extensiveness of gum and tyloses formation. The root system is mechanically blocked and thus rendered physiologically inadequate to supply the plants with water at a time when this substance is in great demand. To what extent the virus alone is able to accomplish this result and how far

micro-organisms and other physiological factors may be concerned in the process, are yet to be determined. It is a matter of common observation that diseased plants will wilt rapidly in the presence of abundant water.

ETIOLOGY

Considerable difficulty was encountered in the determination of the etiology of this disease. From the symptoms, development of wilting, field distribution, spread, etc., it appeared logical to suspect a fungus or bacterial pathogen. Accordingly, numerous isolation experiments were conducted in which various techniques, parts of the plant, degree of infection, early and late symptoms, media, etc., were used. Although the sum total of organisms isolated was large, no one organism ever appeared consistently either in series or from time to time. Among the various organisms isolated were: Bacteria, *Fusarium* spp., *Neocosmospora* sp., *Aspergillus* spp., *Penicillium* spp., *Sclerotium rolfsii* Sacc., *Rhizoctonia solani* Kühn, *Macrophomina phaseoli* (Maubl.) Ashby, and *Pythium butleri* Subr. Representatives of all isolates were inoculated separately into the roots and hypocotyls of young bean plants, and were also used on appropriate media (mostly sterilized oats) to infest sterile soil into which beans were subsequently planted. All the work was carried out under controlled conditions in the greenhouse. Of the organisms used, those pathogenic to beans always produced characteristic symptoms, but in no case did the resulting disease display the symptoms of the black root disease. As a final effort to find a pathogenic organism, certain bean root nodules which appeared somewhat atypical were crushed in sterile water and injected into the hypocotyls of healthy plants. This inoculum likewise failed to produce symptoms of the black root disease.

Because of the peculiar distribution of organisms in the plant parts used in the isolation work, and their sporadic appearance on all the plant parts (except from the pods and from the young seed removed aseptically from fresh diseased pods, which consistently remained sterile in the isolation work), it was thought advisable to try some gross inoculation work. Accordingly, soil was potted directly from beneath dead and dying bean plants in the field, and bean seed was planted in it immediately. This soil contained large quantities of broken, diseased roots, nodules, etc. In conjunction with this experiment, composted, sterile soil was thoroughly mixed with freshly cut, diseased plant parts, including pods, stems, leaves, and roots. Bean seeds were planted in this soil immediately after mixing. All pots were covered with insect cages before or just after the seedlings began to emerge, and the pots were subsequently watered from the bottom so that the cages would not need to be opened. In no instance did any of the plants develop disease symptoms like those of the black root disease. In fact, the only disease that did develop was due to *Pythium butleri*.

TRANSMISSION

The nature and the number of the inoculation trials with micro-organisms, all of which failed to produce the disease under the conditions of the experiments, led the writer to the conclusion that the disease is due to a virus. The following experiments were accordingly conducted to determine the mode of transmission.

Several sweepings of diseased and adjacent bean plants were made in the field, and from 3 to 12 insects of 8 different genera were trapped. These insects were brought immediately into the laboratory, separated, and caged by species on separate healthy young bean plants growing in sterilized composted soil. The insects [5] so tested as possible vectors were: Spotted cucumber beetle (*Diabrotica duodecimpunctata* (Fab.)); bean leaf beetle (*Cerotoma trifurcata* (Forst.); three-cornered alfalfa hopper (*Stictocephala festina* (Say)); Rapid plant bug (*Adelphocoris rapidus* (Say)); tarnished plant bug (*Lygus pratensis* (L.)); a broad-headed bug (*Coriscus pilosus* (H.-S.)); a member of the chinch bug family (*Geocoris punctipes* (Say)); and a small brown leafhopper, not determined.

With the exception of one doubtful case, none of the insects transmitted the disease under the conditions of the trials. In this case, out of several plants infested with the tarnished plant bug, a single plant developed symptoms characteristic of the disease. Although this single case may be significant, the presence of the disease might be explained on the basis of an infected seed. At least the experiment must be repeated with large numbers of both plants and insects before it can be stated definitely that the tarnished plant bug is a vector of the disease.

In conjunction with the insect studies, various types of grafts were tried in an effort to transmit the disease, but owing to the difference in ages of the scion and host tissues, or to technique, no union of tissues resulted. These unsuccessful grafts likewise failed to transmit the disease. In certain cases, when fresh diseased tissue was inserted under the bark and into wounds in the hypocotyl of healthy young plants, discoloration of the vascular tissues was evident for a few centimeters beyond the wound after a week; but the experiment had to be discontinued before completion because of a serious infestation of white fly (*Aleurodes* sp.).

The only successful artificial transmission of the disease was accomplished by hypodermic injections of juice from fresh, diseased pods. The pods in the snap-bean stage were brought to the laboratory and immediately crushed in a sterile porcelain mortar with a porcelain pestle. The juice, undiluted, was filtered through several thicknesses of cheesecloth to remove the plant parts, poured into a glass hypodermic syringe, and injected immediately into the hypocotyls and stems of young, healthy, caged bean plants in the greenhouse.

When injected on June 26, 1939, the bean plants were just showing the first compound leaf. On July 10, flower buds were beginning to form and the vascular system of the plants showed faint discolorations which extended 2 to 3 inches above and below the lowest and the highest of the needle punctures. On July 17, certain inoculated plants were showing fairly intense vascular discolorations up through the second compound leaf and faint vascular discolorations almost to the tip of the plant. Meanwhile, vascular discolorations had progressed downward to the base of the taproot. Although at this time the plants were not permanently wilted, certain leaves showed an incipient wilt, and the lower leaves exhibited symptoms typical of those of diseased plants in the field. A week later, the plants began to die. The leaves wilted and dried out in typical fashion, but for

[5] The writer is indebted to T. L. Bissell, station entomologist, for the identification of the insects.

some reason as yet unknown to the writer, vascular discoloration never reached the intensity of typical field specimens. Greenhouse conditions with the attendant etiolation of the test plants was no doubt a considerable factor in this discrepancy.

Attempted isolations from these inoculated plants showed no more evidence of bacterial or fungus pathogens than did diseased field plants. This phase of the work is being continued, as are also certain phases of the studies on pathological anatomy, in the hope that more information may be obtained on these two critical phases of the disease.

As regards natural transmission of the disease, the writer conducted an experiment the results of which appear highly significant.

On July 11, 1938, 25 seeds, which proved to be viable, were taken from diseased pods of a hybrid pole variety and planted in sterile soil in pots in the greenhouse. Shortly after the seedlings began to emerge, the pots were removed out of doors on account of the high temperature in the greenhouse. On September 2 reddish-brown streaks had appeared near the nodes on about 10 percent of the plants, and when the cortex was peeled back vascular discolorations were found which were identical with those observed on diseased plants in the field. The leaves arising from those areas showing streaks wilted and died. At the time of the last observation before death of the plants, they had not wilted permanently. Other work demanded the writer's attention at this time so that the course of the disease could not be followed in detail. However, the plants were seen again at a later date. At that time they were dead, or very nearly so, and in this condition appeared identical with diseased field plants at a comparable stage. The pods likewise showed typical symptoms, and a few seeds were saved.

Seed-transmission studies are being continued. Seeds are being used from the plants mentioned above in an attempt to determine, relatively, how many generations the virus can be transmitted through the seed.

HOST RANGE

Thus far, because of the difficulty of transmission and the long incubation period of the pathogen, the host range has been determined from observations on natural infections only.

The disease was first seen in the writer's breeding plots on the third generation progeny of Wisconsin Refugee×Michella bean. It was next observed within a few days in a variety trial at Tifton, Ga.; this time on a commercial variety (Black Valentine pole). The seed of this variety had been secured the previous season from a commercial source. Subsequently, the disease was observed on practically all the commercial varieties in the writer's variety trials and on numerous segregating progenies. Specific notes have been taken on the course of the disease on Black Valentine pole, Black Valentine (bunch), Wisconsin Refugee, and White Kentucky Wonder (U. S. No. 3).

In addition to these snap-bean varieties, the writer has also found the disease on several naturally infected dwarf lima beans. In this case the seed was two seasons removed from the breeder. Unfortunately, the writer did not see the planting the previous year so that he is unable to state whether the disease was present on the plants the first year from the breeder.

DISCUSSION

Of the virus diseases of snap beans that have been reported in the literature, those caused by *Datura* virus 1, *Nicotiana* virus 12, and *Cucumis* virus 408 (substrain) perhaps most closely approximate the disease herein described. Whether any relationship exists between any of these viruses and that of black root is not known. The literature on *Datura* virus 1 on beans is very meager and the symptoms have not been described in sufficient detail to enable one to make an accurate comparison.

The symptoms produced by *Nicotiana* virus 12 on snap beans, as they have been described differ in several important respects from the symptoms of the black root disease as they have been observed by the writer. For example, the writer has never observed necrotic areas on the leaflets or sunken necrotic lesions on the stems in plants showing typical symptoms of black root; yet these appear to be cardinal symptoms of the disease caused by *Nicotiana* virus 12. Moreover, vascular discoloration of the pod, a very prominent symptom of black root, has not been described in connection with the former disease.

Although *Cucumis* virus 408 (substrain) produces some vascular discoloration of the root and lower hypocotyl, this symptom is apparently not prominent enough to be regarded as a descriptive symptom of the disease; nor does it appear to extend throughout the vascular system of the plant as in the case of black root.

Even though none of the diseases caused by viruses in other taxonomic categories produce symptoms on the snap bean identical with those of black root, several produce symptoms that are very similar. It is, therefore, evident that much cross-inoculation work must be done in order to determine whether or not any actual relationship exists between the viruses of one or more of these diseases and that of black root.

A very striking feature of black root, as previously mentioned, is the correlation between the stage of maturity of the host and the destructiveness of the disease. The writer has never found typical symptoms of black root in the field earlier than the blossom or young podding stage of the host, although he has searched diligently through thousands of individual plants. It is not reasonable to suppose that the plants are susceptible only at this stage of their development. Numerous field observations, inoculation trials, and the instance cited of the apparent seed-borne nature of the pathogen indicate an extended incubation period.

Whether the entire period intervening between inoculation and the time at which typical disease symptoms are evident may properly be termed an incubation period in the case of a virus disease is open to question. Certainly no other virus disease of snap beans, so far as the writer knows, has been reported as having such an extended incubation period.

It is, of course, entirely conceivable that the symptoms exhibited by wilting and dying plants may in a sense be secondary manifestations of physicochemical changes only indirectly related to those which we visualize as virus activity. A more definite statement of this viewpoint must await the results of later research on certain details of the problem now under consideration.

Whether the symptoms expressed by the writer in terms of "vascular discoloration" are in the same category as the symptoms on the bean

and on other plants affected by viruses and by various virologists termed "necroses," may, perhaps be a matter or interpretation. Insofar as the studies on the pathological anatomy of black root have extended, there is no evidence of tissue break-down or collapse, the usual physical signs of death of tissues. As stated previously, most of the discoloration is residual in the walls of the vascular elements; although in instances of abundant tyloses or gum formation, the cell contents are discolored. It is hoped that a more extensive study of the pathological anatomy of the disease will clarify the situation.

SUMMARY

What appears to be a new disease of snap beans in the vicinity of Experiment, Ga., is described.

The symptoms consist at first of an incipient wilt of a part of the plant, followed by permanent general wilt of all the leaves and death; although under certain conditions parts of the plant may continue to live for a short time. Accompanying these symptoms is an extensive discoloration in the vascular system of the root, stem, leaves, and pods. The lower leaves exhibit a faint but distinct checkerboard pattern of light and dark areas.

The disease has been transmitted by hypodermic injections of fresh juice from diseased pods in the snap bean stage and through viable seed from diseased pods.

Thus far, experiments with insect transmission have given negative results except possibly in one instance in which the tarnished plant bug (*Lygus pratensis*) was used.

The host range as thus far determined includes several varieties of bunch and pole beans, as well as a variety of lima beans.

Work is being continued on certain phases of pathological anatomy, transmission, virus characteristics, and host range.

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